BIOTECHNOLOGICAL PRODUCTION AND CHARACTERIZATION OF SOPHOROLIPIDS FROM *Candida bombicola* MTCC 1910

Dissertation submitted to

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Chennai

In partial fulfillment for the award of degree of

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(PHARMACEUTICAL BIOTECHNOLOGY)

Submitted by
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Under the guidance of
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COLLEGE OF PHARMACY

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COIMBATORE – 641044

CERTIFICATE

This is to certify that the dissertation entitled **"BIOTECHNOLOGICAL PRODUCTION AND CHARACTERIZATION OF SOPHOROLIPIDS FROM *Candida bombicola* MTCC 1910"** *was carried out by* **P.SIVACHIDAMBARM** *in the Department of Pharmaceutical Biotechnology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore which is affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, under my direct supervision and guidance to my fullest satisfaction.*

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1. INTRODUCTION

Humans have transformed the Earth and sustained population growth will further accentuate the influence of humans on the environment. As a result, concern about environmental protection has increased recently from a global viewpoint and the chemical and pharmaceutical industries anticipate revolutionary transformations in the future. Many industries have however recognized the potential of living cells in the pre-treatment of raw materials, processing operations, product modifications, selective waste management, energy recycling and conservation[1, 2]. It is also becoming increasingly recognized that surfactants can have a range of uses. The total quantity of chemical and biological surfactants produced in the US and all over the world is estimated at more than 10 billion pounds and 25 billion pounds, respectively. In spite of the fact that there are so many chemical surfactants, there is great interest in biosurfactants, as they may provide new properties that the classical chemical surfactants may lack[3].

1.1. GENERAL INTRODUCTION ABOUT SURFACTANTS

In any heterogeneous system, boundaries are of fundamental importance to behaviour of the system as a whole. Surfactants are substances that adsorb to and alter the conditions prevailing at the interfaces. Emulsifiers are a subclass of surfactants that stabilize the dispersion of one drop in another. Eg oil water emulsions. The reason that surfactants are amphiphilic compounds containing both hydrophobic (nonpolar) and hydrophilic (polar) moieties that confer ability to accumulate between fluid phases such as oil/water or air/water, reducing the surface and interfacial tensions and forming emulsions[4]. The distinguishing character of surfactant molecule is due to their mixed hydrophilic and hydrophobic nature. They are able to form either micelles or reversed micelles, or aggregates to form rod shaped micelles, bilayers and vesicles. This dynamic process is based on the ability of surfactant to reduce surface tension by governing the arrangement liquid molecules, thus influencing the formation of hydrogen bonds and other hydrophilic – hydrophobic interactions[5]. These surface activity properties make

surfactants as one of the most important and versatile class of chemical products, used on a variety of applications.

1.2. BIOSURFACTANTS

Biosurfactants (BS) are amphiphilic compounds produced on living surfaces, mostly on microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension (ST) and interfacial tensions between individual molecules at the surface and interface, respectively. Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon (C_xH_y), microorganisms facilitate their diffusion into the cell by producing a variety of substances, the biosurfactants[6].

They have been known for over 40 years but rapid advances in biotechnology over the past decade have led to considerable interest in the development of biological methods for manufacturing of biosurfactants has steadily increased. Microorganisms, because of their large surface-to-volume ratio and diverse synthetic capabilities are promising candidates for widening the present range of surfactants[7]. Numerous strains of microorganisms which produce biosurfactants include many of yeasts, bacteria, and filamentous fungi.

1.3. CLASSIFICATION OF BIOSURFACTANTS

Biosurfactants constitute a diverse group of surface-active molecules and are known to occur in a variety of chemical structures. Synthetic surfactants are usually classified according to the nature of their polar groups but biosurfactants are generally classified on the basis of their biochemical nature. Generally biosurfactants can be divided into low-molecular-mass molecules, which efficiently lower surface and interfacial tension, and high-molecular-mass polymers, which are more effective as emulsion stabilizing agents. The major classes of low-mass surfactants include glycolipids, lipopeptides and phospholipids, whereas high mass includes polymeric and particulate surfactants[8]. Most biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long-chain fatty acids or fatty acids derivatives whereas the hydrophilic

portion can be a carbohydrate, amino acid, phosphate or cyclic peptide. Table 1 shows the major biosurfactant classes and the microorganisms involved. Bacteria are the predominant group of surfactant producing organisms however some yeast species are also involved. A brief discussion about each class of biosurfactant is given below.

1.3.1. Glycolipids [7]

These are compounds of a carbohydrate and a lipid; the linkage is by way of either ether or an ester group. The main glycolipids which are found to occur and are most often investigated are:

- Rhamnolipids
- Trehalose lipids
- Sophorolipids
- Monnosylerythitol lipids

Table 1

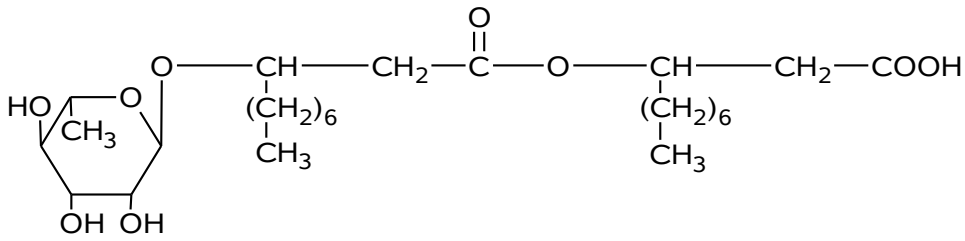
Major biosurfactant classes and the micro organisms involved

Surfactant class	Microorganism
Glycolipids	
Rhamnolipids	*Pseudomonas aeruginosa*
Trehalose lipids	*Rhodococcus erithropolis* *Arthobacter sp.*
Sophorolipids	*Candida bombicola, Candida apicola*
Mannosylerythritol lipids	*Candida antartica*
Lipopeptides	
Surfactin/iturin/fengycin	*Bacillus subtilis*
Viscosin	*Pseudomonas fluorescens*
Lichenysin	*Bacillus licheniformis*
Serrawettin	*Serratia marcescens*
Phospholipids	*Acinetobacter sp.,* *Corynebacterium lepus*
Surface active antibiotics	
Gramicidin	*Brevibacterium brevis*
Polymixin	*Brevibacterium polymyxa*
Antibiotic TA	*Myxococcus xanthus*
Fatty acids/neutral lipids	
Corynomicolic acids	*Corynebacterium insidibasseosum*
Polymeric surfactants	
Emulsan	*Acinetobacter calcoaceticus*
Alasan	*Acinetobacter radioresistens*
Liposan	*Candida lipolytica*
Lipomanan	*Candida tropicalis*
Particulate biosurfactants	
Vesicles	*Acinetobacter calcoaceticus*
Whole microbial cells	*Cyanobacteria*

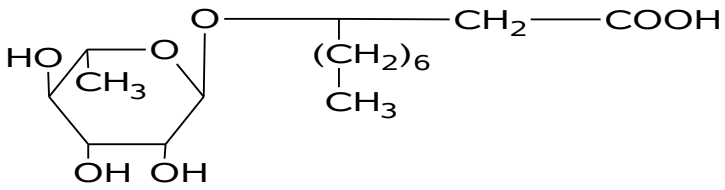
1.3.1.1. Rhamnolipids

Rhamnolipids are glycolipids which contain one or two molecules of rhamnose linked to one or two molecules of β – hydroxy decanoic acid (Fig.1). While the OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, the OH group of the second acids is involved in ester formation. Since one of the carboxylic acid is free, the rhamnolipids are anions above pH 4.0[6].

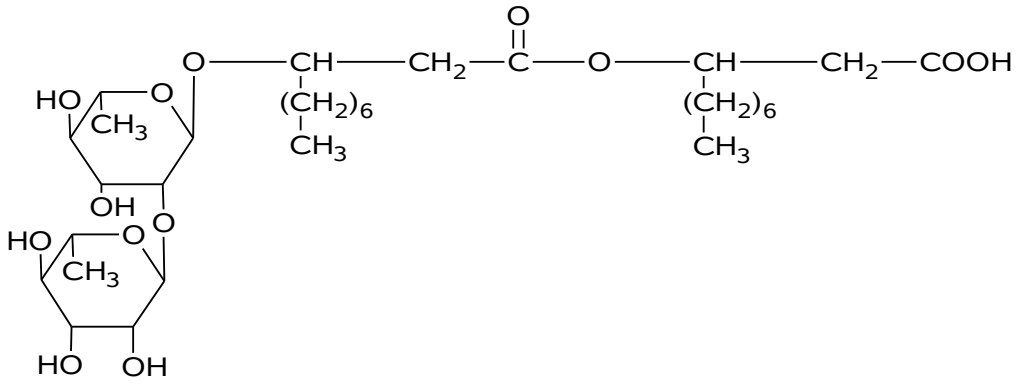
Several species of *Pseudomonas*, over the year have been shown to produce a variety of rhamnolipids[9]. Four different forms of rhamnolipids synthesized by *Pseudomonas* DSM 2659[10]. The purified rhamnolipids from *Pseudomonas sp*. lower the interfacial tension between water and n-hexadecane to around 1mN/m and surface tension of water to 25-30 mN/m from 72 mN/m[6].



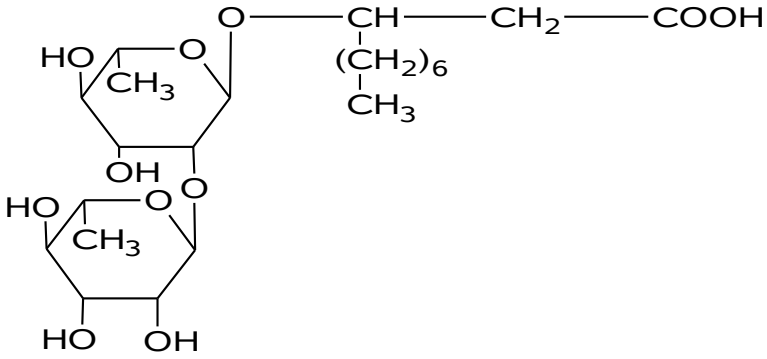
Rhamnolipid 1



Rhamnolipid 2



Rhamnolipid3



Rhamnolipid 4

Fig. 1 Structures of rhamnolipid

1.3.1.2. Trehalose lipids

Trehalose lipids are usually disaccharide trehalose molecules linked at C_6 and C_6' to mycolic acids (Fig.2). They are usually associated with the cell wall structure of most species of the genera *Mycobacterium*, *Nocardia* and *Corynebacterium*. Mycolic acids are long-chain α branched β-hydroxy fatty acids and the chain synthesized is a characteristic of a genera. Trehalose lipids can be either trehalose mycolates or trehalose esters[6]. A biosurfactants from *Arthrobacter paraffineus* KY 4303 when grown on n-paraffin as the sole source and this biosurfactant was an α- branched β- hydroxyl fatty acid trehalose ester[11].

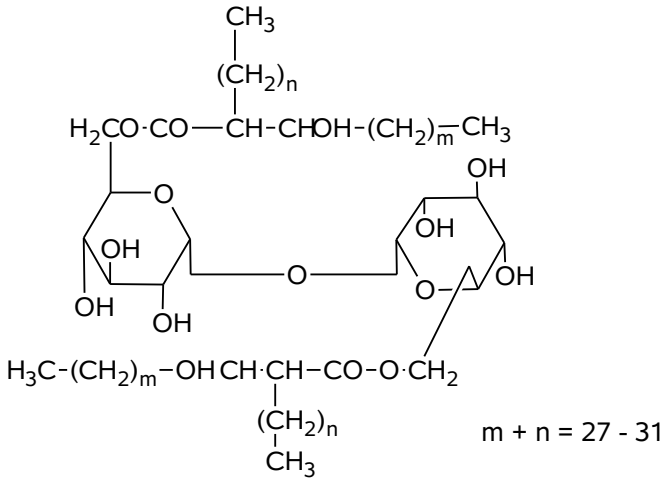
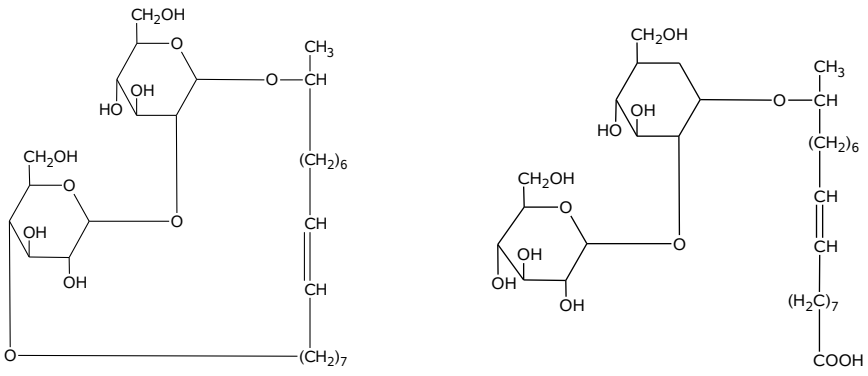


Fig. 2 Structure of trehalose lipid

1.3.1.3. Sophorolipids

Sophorolipids are biosurfactants which consists of a dimeric carbohydrate sophorose linked to long-chain hydroxy carboxylic acids by glycosidic linkage (Fig. 3). Generally sophorolipids occurs as mixture of macrolactones and free acid form. Yeast has been shown to be potent producers of sophorolipids as an extra cellular biosurfactant. *Torulopsis bombicola* produces a sophorolipid like biosurfactant during alkane fermentation[12] while *Torulopsis petrophilum* and *Torulopsis apicola* are major producers *o*f sophorolipids[13, 14].



Lactone Form **Acid Form**

Fig. 3: Structure of lactonized and free acid form of sophorolipids

1.3.2. Phospholipids

These are the esters formed between the alcohol groups on a lipid and a phosphate[7].

Phospholipids are major components of microbial membranes. When certain C*x*H*y*-degrading bacteria or yeast[6] are grown on alkane substrates, the level of phospholipids increases greatly. *Thiobacillus thioxidans*[15] produces a significant amount of phospholipids and also *Aspergillus* sp grown on hydrocarbon produces phospholipids biosurfactant [16].

1.3.3. Fatty acids

The fatty acids produced from alkanes by microbial oxidations have received maximum attention as surfactants. Besides the straight-chain acids, microorganisms produce complex fatty acids containing OH groups and alkyl branches. *Corynebacterium insidibasseosum* produces significant amount of Corynomicolic acids,a fatty acid type , complex acid biosurfactant[6].

1.3.4. Lipopeptides and lipoproteins [9]

These consist of a lipid attached to a polypeptide chain. The lipopeptides which are found to occur are

- Surfactin
- Lichenysin
- Viscosin
- Serrawettin

1.3.4.1. Surfactin

One of the most active biosurfactants produced by *Bacillus subtilis* is a cyclic lipopeptide surfactin[17]. It is composed of seven amino acid ring structure coupled to fatty acid chain via lactone linkage (Fig. 4). It lowers the surface tension of water from 72 mN/m to 29.9 mN/m[5]

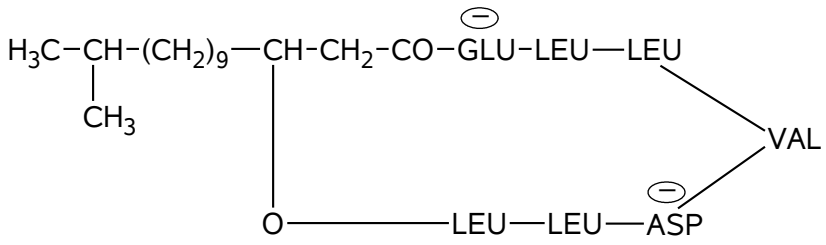


Fig. 4 Structure of surfactin

1.3.4.2. Lichenysin

A biosurfactant produced by *Bacillus licheniformis* is lichenysin with a lipophilic fatty acid moiety joined via lactone linkage to the hydrophilic peptide ring structure, which is similar in structural and physiochemical properties to the surfactin[5]

1.3.5. Polymeric surfactants

These are products again formed between saccharide units and fatty acid residues; but they are polymeric in nature[9].

- Emulsan
- Liposan
- Alasan
- Lipomanan

1.3.5.1 Emulsan

Oil degrading bacterium *Acinetobacter calcoaceticus RAG-1 (ATCC 31012)* produces a potent extracellular, high molecular weight polymeric biosurfactant called emulsan[18].It has been characterized as a poly anionic amphipathic, hetropolysaccharide with a molecular weight of 10^6. The hetropolysaccharide back bone consists of repeating trisaccharides of N-acetyl –D –galactosamine, of N-acetyl galactosamine uronic acids and un identified N-acetyl amino sugars[19]. It works at low concentration as 0.0001-0.1%.

1.3.5.2. Liposan

The extracellular emulsifier called liposan which is produced by *candida lipolytica* is inducible in nature and is primarily (97-98%) composed of

carbohydrates portion is a hetropolysacharide consisting of glucose, galactose, galactosamine and galacturonic acids[20].

1.3.6. Surface active antibiotics

- Gramicidin S
- Polymixins
- Antibiotic TA

1.3.6.1.Gramicidin S

Many bacteria produce a cyclosymmetric decapeptide antibiotic, gramicidin S. Spore preparations of *Brevibacterium brevis* contain large amounts of gramicidin S bound strongly to the outer surface of the spores[21.] The antibacterial activity of gramicidin S is due to its high surface activity[22].

1.3.6.2. Polymixins

These are a group of antibiotics produced by *Brevibacterium polymyxa* and related bacilli. Polymixin B is a decapeptide, a branched chain fatty acid is connected to the terminal 2, 4-diaminobutyric acid (DAB). Polymixins are able to solubilize certain membrane enzymes[23].

1.3.6.3. Antibiotic TA

Myxococcus xanthus produces antibiotic TA which inhibits peptidoglycan synthesis by interfering with polymerization of the lipid disaccharide pentapeptide[24]. Antibiotic TA has interesting chemotherapeutic applications[25].

1.3.7. Particulate biosurfactants

- Extracellular vesicles
- Whole microbial cells

1.3.7.1 Extracellular vesicles

Accumulation of extracellular membrane vesicles having 20-50 nm diameters has been reported in having *Acinetobacter* sp. H01-N with a density of 1.158 g/cm[3, 26].

1.3.7.2 Whole microbial cells

Most hydrocarbon-degrading microorganisms, many nonhydrocarbon degraders, some species of *Cyanobacteria*[27], and some pathogens have a strong affinity for hydrocarbon-water and air-water interfaces. In such cases, the microbial cell itself is a surfactant.

1.3.8. New class of biosurfactant

Bodour *et al.* (2004) [28] have recently described a new class of biosurfactants named flavolipids produced by a soil isolated *Flavobacterium* sp. The new surfactant showed strong surface activity and emulsifying ability, and exhibits a polar moiety that features citric acid.

1.4. PROPERTIES OF BIOSURFACTANTS

The main distinctive features of biosurfactants are their surface activity; tolerance to pH, temperature and ionic strength; biodegradability; low toxicity; emulsifying and demulsifying ability. A very brief description of each property is given below.

1.4.1. Surface and interface activity

A good surfactant can lower surface tension (ST) of water from 72 to 35 mN/m and the interfacial tension (IT) of water/hexadecane from 40 to 1 mN/m[29]. Surfactin from *Bacillus subtilis* can reduce ST of water to 25 mN/m and IT of water/hexadecane to <1 mN/m[30]. The rhamnolipids from *Pseudomonas aeruginosa* decreased ST of water to 26 mN/m and IT of water/hexadecane to value <1 mN/m[31]. The sophorolipids from *Candida bombicola* were reported to reduce ST to 33 mN/m and IT to 5 mN/m[32]. In general, biosurfactants are more effective and efficient and their CMC (critical micelle concentration) is about 10-40 times lower than chemical surfactants, i.e., less surfactant is necessary to get a maximal decrease on ST[33].

1.4.2. Temperature, pH and ionic strength

Many biosurfactants and their surface activity are not affected by environmental conditions such as temperature and pH. McInerney, Javaheri, and Nagle (1990)[34] reported that lichenysin from *Bacillus licheniformis* JF-2 was not affected by temperature (up to 50 ºC), pH (4.5-9.0) and by NaCl and Ca concentrations up to 50 and 25 g/L, respectively. A lipopeptide from *Bacillus subtilis* LB5a was stable after autoclave (121 ºC/20 min) and after 6 months at -18ºC; the surface activity did not change from pH 5 to pH 11 and NaCl concentrations up to 20%[35].

1.4.3. Biodegradability

Unlike synthetic surfactants, microbial-produced compounds are easily degraded[36] and particularly suited for environmental applications such as bioremediation[29,37].

1.4.4. Low toxicity

Few data are available in literature regarding the toxicity of microbial surfactants, they are generally considered low or non-toxic products and therefore, appropriate for pharmaceutical, cosmetic and food uses. A report suggested that a synthetic anionic surfactant (Corexit) displayed a LC50 (concentration lethal to 50% of test species) against *Photobacterium phosphoreum* 10 times lower than rhamnolipids, demonstrating the higher toxicity of the chemical-derived surfactant[38]. These authors also reported that biosurfactants showed higher EC50 (effective concentration to decrease 50% of test population) values than synthetic dispersants[39]. A biosurfactant from *Pseudomonas aeruginosa* was compared with a synthetic surfactant (Marlon A-350) widely used in industry in terms of toxicity and mutagenic properties. Both assays indicated the higher toxicity and mutagenic effect of the chemical-derived surfactant whereas biosurfactant was considered slightly to non-toxic and non-mutagenic[40].

1.4.5. Emulsion forming and emulsion breaking

Stable emulsions can be produced with a life span of months and years[41]. Biosurfactants may stabilize (emulsifiers) or destabilize (de-emulsifiers) the emulsion. High-molecular-mass biosurfactants are in general better emulsifiers than low-molecular mass biosurfactants. Sophorolipids from *Torulopsis bombicola* have been shown to reduce surface and interfacial tension but not to be good emulsifiers[32]. By contrast, liposan has been shown not to reduce surface tension but used successfully to emulsify edible oils[42]. Polymeric surfactants offer additional advantages because they coat the droplets of oil, thereby forming very stable emulsions that never coalesce. This property is especially useful for making oil/ water emulsions for cosmetics and food.

1.5. BIOSURFACTANT PRODUCTION

The production of biosurfactants by microorganisms can be during exponential growth or it may be during the stationary phase of growth when the nutrient limiting conditions start prevailing in the growth medium. In case of growth associated biosurfactant production, these exists a parallel relationship between substrate utilization, growth and biosurfactant production. The production of AP-6, a glycoprotein type of biosurfactant by *Pseudomonas fluorescens* 378 is growth associated. Similarly, the production of emulsan in *Arthrobacter caloaceticus* and the fermentative production of surface active agent from *Bacillus aereus* IAF-346 and *Bacillus* species IAF 343 were found to be growth associated. The production of rhamnolipids by *Pseudomonas aeruginosa* is also related to growth[5].

1.6. MECHANISM OF BIOSYNTHESIS[5]

In general, growth and microbial surfactant production proceed as separate events. In the exponential phase of growth there is often only a very low rate of surfactant production, overproduction of the surfactant then occurs as the cells cease to grow. Hommel and Ratledge have proposed a theory that a small amount of microbial surfactant is needed for alkane solubilization and uptake. According to them, surfactant is not consumed by cells in the uptake process. Like the production of other metabolites, once the signal to start surfactant production has

been given by the presence of alkane, then surfactant production will continue in an unregulated manner till the signal to stop is received. As that signal the disappearance of the last alkane droplet never arrives, the cells continue surfactant production even after they have ceased to grow. The emulsification of the residual alkanes by the surfactant must continue beyond what the cells require but the cells have no means of halting this needless production of the surfactant. Cells do try to regulate this by feedback inhibition of the intracellular pool of fatty acyl-CoA, but still, at the end of growth, cells are found with excess of unused alkane within them.

The physiology of a microorganism is not only determined by its genetic information, but is also a function of environmental conditions. High microbial surfactant production, which is a phenotypic response, is found to be characteristic under certain qualitative and quantitative environmental conditions. Production of microbial surfactants by microbes in general is dependent on nutritional factors and environmental factors.

1.7. PHYSIOLOGICAL ROLE OF BIOSURFACTANTS

Microorganisms require energy, reducing power, and precursors to synthesize the molecular components which are essential for growth and reproduction. Though, the main physiological role of biosurfactants is to permit microorganisms to grow on water- immiscible substrates by reducing surface tension at a phase boundary thus making the substrate more readily available for uptake and metabolism, the molecular mechanism of the uptake process of these substrates are, however, still not clear[5].

1.8. NUTRITIONAL FACTORS INFLUENCING PRODUCTION OF BIOSURFACTANTS

1.8.1. Effect of carbon source

Innumerable researches over the years have shown the preference of microorganisms for hydrocarbon substrates for biosurfactant production to carbohydrate ones. There is a sudden burst in the production of biosurfactants whenever microbes grow at the expense of water immiscible substrates. Singh and Desai[43] have shown hydrocarbon emulsification by *Candida tropicalis* during their cultivation on hexadecane and oily wastes but there was no emulsifier production when the yeasts were grown in a glucose medium.

Earlier, Rapp *et al* [44] had studied the formation of trehalose lipids by *Rhodococcus erythropolis* which was induced by alkanes with chain lengths between C_{12} and C_{18} (yield being 2.1g/L).

Candida lipolytica produces an inducible extracellular emulsification activity when it is grown with a number of water immiscible carbon substrates, but negligible emulsification activity was produced by this yeast when it was grown on glucose as the carbon substrate [45].

In *Arthrobacter paraffineus* KY 430, Suzuki *et al* [11] isolated trehalose lipids when grown on paraffin as the carbon source. *Corynebacterium lepus* produces a lipolytic biosurfactant with surface active properties when grown with kerosene as the carbon source [46]. Patel and Gopinathan[47] isolated two *Bacillus* strains capable of producing a bioemulsifier when grown on immiscible organophosphorus pesticides as substrate. *Pseudomonas aeruginosa*, when grown on n-alkanes,secretes surface active substances in the medium , but these products are not detected during growth on carbohydrates [9].

There are instances when certain bacteria prefer one type of hydrocarbon substrate to the other, i.e. some produce biosurfactants when grown only on short chain alkanes, while others on long chain alkanes or vegetable oils, or crude oil only.

Not only hydrocarbons are used as carbon substrates for production of biosurfactants, there are certain species of bacteria which produce biosurfactants when grown on carbohydrates and not on hydrocarbons.

Surfactin, the most potent biosurfactant produced by *Bacillus subtilis* is synthesized when it is grown on glucose as the carbon source and its production is inhibited when grown on hydrocarbons[30]. *Pseudomonas fluorescens* 378 produces a novel surface active compound called AP-6 when grown on sucrose as the carbon source[48].

Literature provides examples of one carbon source being used for the growth and the other for the production of surface active compounds. *Arthrobacter paraffeinus* ATCC 19558, when growing on glucose and supplemented with hexadecane in the medium during stationary phase of growth, causes significant production of biosurfactant[49],

Corynebacterium lepus produced a large amount of biosurfactant when grown on glucose, but it remains cell bound. The surfactant is released from the cells when treated with hexadecane[50].

When a complex medium containing a carbohydrate, i.e. glucose and vegetable oil are used, *Torulopsis bombicola* gives a biosurfactant yield of 70g/L[51].

There was a surge in production of glycolipid with addition of glucose after the culture has reached an exponential growth with vegetable oil. Analogous results were obtained when initial fermentation medium contained only glucose, and vegetable oil was added after growth was over.

1.8.2. Effect of nitrogen source

Other than the carbon source to be used for substrate, the type and addition of nitrogen source in the growth medium also influences the production of biosurfactants. Yeast extracts, peptone and bactotryptone have also been shown to have a positive effect on biosurfactant production by several workers[52,53].

The influence of various nitrogen sources on glycolipid production by *Nocardia cornebacteroids* was examined by Powalla and co-investigators and they found that inorganic nitrogen sources gave distinctly higher yields of biosurfactants of which $NaNO_3$ gave the highest production. *Pseudomonas aeruginosa* gives a high yield of biosurfactant when grown on glucose as the carbon source and nitrate is used as the nitrogen source but the yield declines drastically when yeast extract is added to the medium[54].

Enhanced surfactant production by *Bacillus subtilis* was achieved by use of NH_4NO_3 as nitrogen source while neither NH_4Cl nor $NaNO_3$ as nitrogen source could individually enhance biosurfactant production[55]. There have been many reports where nitrogen limiting conditions have been correlated with biosurfactant yields Emulsification activity is more in nitrogen limiting growth conditions than in substrate limiting conditions in *Candida tropicalis*[56]. In *Pseudomonas* 44TI[57] the production of biosurfactant soon after the culture reached a nitrogen limiting stage.

1.8.3. Effect of metal cations

Other than nitrogen limitation, a minimized concentration of respective salts of magnesium, calcium, potassium, sodium and trace-elements yielded high biosurfactant production[54].

Mulligan *et al* [58] have shown that in a phosphate limited medium, protease / peptone / glucose / ammonium salts (PPGAS), supported the best yield of biosurfactant by *Pseudomonas aeruginosa*. But in PPGAS medium *Pseudomonas aeruginosa* produces only the biosurfactant and no emulsifier.

Iron limitation is known to stimulate the production of several extracellular secretions and biosurfactant is one of them[59]. In contrast, surfactin production by *Bacillus subtilis* is found to be stimulated by addition of iron and manganese salts to the medium[30]. The yield improved considerably from 4 to 9 g/l by addition of either iron or manganese salts. When sterile $FeSo_4$ was added to fermenter after stationary period has been achieved there was a second dramatic growth phase and production of surfactin. Same effect was observed after the addition of $Fecl_2$. A similar study with $Mnso_4$ also resulted in a second burst of growth on surfactin production.

1.8.4. Effect of other chemicals

There are various reports proving an increase in the biosurfactant production by the addition of certain chemicals such as antibiotics, metal chelating agents and growth stimulants. Many researchers have shown that there is increased production of biosurfactants, just by addition of metal chelators like EDTA into the growth medium[60].It is based on the simple principle that EDTA chelates the metal ions in the medium, thus bringing about metal limitations resulting in increased production of biosurfactants.

An accelerated release of emulsan from the cell surface of *Arthrobacter calco aceticus* RAG occurs when early log phase cells are incubated in the presence of inhibitors of protein synthesis such as chloramphenicol[61].

Reports show that addition of various surfactants into the hydrocarbon medium has a good effect on the growth of microorganisms. *Acinetobacter calcoaceticus* RAG-1 mutants, isolated by Shabtai and Gutnick[62] produced higher levels of emulsan compared to the parent strain when grown with higher doses of cationic surfactant.

Hisatsuka *et al* [9] isolated a growth stimulant (GS) produced by *Pseudomonas aeruginosa*, which stimulated the growth of *Pseudomonas* on hexadecane medium.

1.9. RENEWABLE SUBSTRATES

1.9.1. Olive Oil Mill Effluent (OOME)

Olive oil extraction involves an intensive consumption of water and produces large amounts of olive oil mill wastewater, thus causing deleterious environmental effects. OOME is a black liquor and consists of a high content of organic matter depending on the olive oil extraction procedure[63]. OOME contains toxic substances such as polyphenols, but also valuable organic substances such as sugars, nitrogen compounds, organic acids and residual oils. Mercade *et al.* [64] found that *Pseudomonas* sp. could reduce the surface tension in culture medium comprising OOME (100 g/l) and $NaNO_3$ (2.5 g/l). Surface-active compounds produced from *Pseudomonas* sp. cultured in OOME medium included rhamnolipids biosurfactant, A total conversion yield was estimated to be 14g of rhamnolipids per kg of OOME after 150 h of cultivation time.

1.9.2. Animal fat

Animal fat and tallow can be obtained at large quantities from meat processing industries and have been used as a cooking medium for foods. Deshpande and Daniels [65] used animal fat for the production of sophorolipid biosurfactant by yeast, *Candida bombicola* when only fat was provided as a sole carbon source, the growth was poor. The mixture of 10% glucose and 10% fat gave the highest level of growth. Sophorolipid was produced at levels of 97 g/l and 12 g/l without and with pH control, respectively.

Haba *et al.* [66] used olive or sunflower cooking oil as carbon source for biosurfactant production by 36 isolated bacteria. Most of the *Pseudomonas* strains tested showed satisfactory growth when cultivated on either used olive oil or used sunflower oil. However, sunflower oil was not as a good substrate as olive oil, either for cell growth or for biosurfactant production. *Pseudomonas* strains decreased the surface tension of the medium to 34-36 mN/m and the emulsions with kerosene remained stable for three months.

1.9.3. Soapstock

Soapstock is a gummy, ambercolored by-product of oilseed processing. It is produced when hexane and other chemicals are used to extract and refine edible oil from the seeds. Shabtai[67] reported the production of two extracellular capsular heteropolysaccharides, emulsan and biodispersan by *Acinetobacter calcoaceticus* RAG-1 and *Acinetobacter calcoaceticus* RAG-2, respectively using soap stock as a carbon source. Emulsan forms and stabilizes oil-water emulsion[68], whereas biodispersan disperses the large solid limestone granules, forming micrometer-size water suspension[69]. Both polysaccharides are synthesized within the cell, exported to their outer surface to form an extracellular cell-associated capsule and released subsequently into the growth medium. After 50 h and 45 h of the fermentation, emulsan and biodispersan at levels of 25g/l and 12 g/l were produced, respectively.

1.9.4. Molasses

Molasses is a co-product of sugar production, both from sugar cane as well as from sugar beet. It is defined as the runoff syrup from the final stage of crystallization, in which further crystallization of sugar is uneconomical. Molasses generally consists of 48-56% total sugar (mainly sucrose), 9-12% non-sugar organic matter, 2-4% protein (N × 6.25), 1.5-5% potassium, 0.4-0.8% calcium, 0.06% magnesium, 0.6-2.0% phosphorus, 1.0-3.0 mg/kg biotin, 15-55 mg/kg pantothenic acid, 2500-6000 mg/kg inositol and 1.8 mg/kg thiamine [70] . Patel and Desai [71] used the molasses and cornsteep liquor as the primary carbon and nitrogen source to produce rhamnolipid biosurfactant from *Pseudomonas aeruginosa* GS3. The biosurfactant production reached the maximum when 7% (v/v) of molasses and 0.5% (v/v) of cornsteep liquor were used. Maximal surfactant production occurred after 96 h of incubation, when cells reached the stationary phase of growth. A rhamnose concentration of 0.25 g/l and a reduction of interfacial tension between surfactant and crude oil of up to 0.47 mN/m were obtained.

1.9.5. Whey

Whey is a liquid by-product of cheese production containing the water soluble components. Dubey and Juwarkar[72] cultivated *Pseudomonas aeruginosa* BS2 on whey waste for biosurfactant production. Within 48 h of incubation the yield of biosurfactant obtained was 0.92 g/l. Strain BS2 produced a crystalline biosurfactant as the secondary metabolites and its maximal production occurred after the onset of nitrogen-limiting conditions. The isolated biosurfactant possessed the potent surface-active properties, as it effectively reduced the surface tension of water from 72 to 27 mN/m and formed 100% stable emulsion of a variety of water insoluble compounds.

1.9.6. Starch-rich wastes

The processing of agroindustrial raw materials such as cassava or potato produces the large amount of waste, whose accumulation leads to environmental pollution. Due to the high amounts of starch or reducing sugar, those wastes have been recognized as a suitable feedstock for industrial fermentations. Potato process effluents (wastes from potato processing industries) were used to produce biosurfactant by *Bacillus subtilis*[73,74]. Cassava wastewater, another carbohydrate-rich residue, which is generated in large amounts during the preparation of cassava flour, is also an attractive substrate and has been used for surfactin production by *Bacillus subtilis*[75]. Several other starchy waste substrates, such as rice water (effluent from rice processing industry and domestic cooking), cornsteep liquor, and wastewater from the processing of cereals, pulses and molasses, have tremendous potential to support microbial growth and biosurfactant production.

Table 2

Use of inexpensive raw materials for the production of biosurfactants by various microbial strains

Low cost or waste raw material	Biosurfactant type	Producer microbial strain
Rapeseed oil	Rhamnolipids	*Pseudomonas species* DSM 2874
Babassu oil	Sophorolipids	*Candida lipolytica* IA 1055
Turkish corn oil	Sophorolipids	*Candida bombicola* ATCC 22214
Sunflower and soybean oil	Rhamnolipid	*Pseudomonas aeruginosa* DS 10
Sunflower oil	Lipopeptide	*Serratia marcescens*
Soybean oil	Mannosylerythritol lipid	*Candida* sp. SY16
Waste frying oils (sunflower and olive oil)	Rhamnolipid	*Pseudomonas aeruginosa* 47T2 NCIB 40044
Soybean soapstock waste	Rhamnolipid	*Pseudomonas aeruginosa* LB
Sunflower oil soapstock waste	Rhamnolipid	*Pseudomonas aeruginosa* LBI
Oil refinery wastes	Glycolipids	*Candida antarctica* and/or *Candida apicola*
Soybean oil refinery wastes	Rhamnolipids	*Pseudomonas aeruginosa* AT10
Curd whey and distillery wastes	Rhamnolipid	*Pseudomonas aeruginosa* strain BS2
Potato process effluents	Lipopeptide	*Bacillus subtilis*

1.10. ENVIRONMENTAL FACTORS INFLUENCING BIOSURFACTANT PRODUCTION

Other than nutrient limitations, many researchers shown the influence of environmental conditions like pH, cultivation temperature, rate of oxygen transfer and speed of agitation is increasing the yield of biosurfactant production.

1.10.1. Effect of pH

According to Santos *et al*., there is a maximum rhamnolipid production at a pH of 6.2-6.4 by *Pseudomonas aeruginosa*[54].The pH of the medium plays an important role in sophorolipid production by *Torulopsis bombicola*[76].In *Bacillus cereus*, lowering the pH of the medium below 6.5 decreased the product, i.e. the biosurfactant yield[77].

1.10.2. Effect of cultivation temperature

Serratia rubidaca shows surface activity only when grown at 30^0C but not at 37^0C temperature[78]. The biosurfactants, rubiwettins R and RG were located in extracellular vesicles which are formed only when the cells are grown at 30^0C. Production of biosurfactant by *Arthrobacter paraffenius* and *Pseudomonas* sp. DSM-2874 is found to be sensitive to changes in temperature. There is maximum rhamnolipid production at an optimal temperature of 32-34^0C by *Pseudomonas aeruginosa*[54]. Interestingly, temperature has been found to alter the composition of biosurfactant produced.

1.10.3. Effect of agitation

Increase in the agitation speed results in the reduction of biosurfactant yield in *Nocardia erythropolis.*

1.10.4. Effect of oxygen transfer

Sheppard and cooper[79] have recently studied the surfactin production in cyclone column reactor by *Bacillus subtilis* and concluded that oxygen transfer is one of the key parameter for process optimization and scale up of surfactin production.

1.11. REGULATION OF BIOSURFACTANT PRODUCTION

All microorganisms require a source of carbon, hydrogen, nitrogen, oxygen and to a smaller degree sulphur and phosphorous for growth. These act as precursors in the biosynthetic reactions of the cells leading to formation of amino acids, fatty acids. etc., which, in turn lead to formation of secondary metabolites by involving either nitrogen, or phosphate metabolism or the process of carbon metabolism. Very little information is known about the synthetic pathway of biosurfactants. The physiology of biosurfactant production has still not been out lined but various attempts have been made to study the process of biosurfactant production in relation to other metabolic pathways of the bacterial cell, but more information is needed to come to a clear picture about the physiology associated with biosurfactant production[5].

De roubin and Mulligan[55] followed the activity of an enzyme in T.C.A. cycle, isocitrate dehydrogenase (E.C. 1.1.1.42) during the production of biosurfactant by *Bacillus subtillis*. Initiation of surfactin production occurred as redox values increased and isocitrate dehydrogenase activity decreased (i.e., increased 'surfactin' yield correlated with low levels of enzyme activity). In the same study they got a mutant which suppressed the activity of isocitrate dehydrogenase over the parent strain but simultaneously produced 3 1/2 times more surfactin.

1.12. MUTANT AND RECOMBINANT STRAINS

The genetics of the producer organism is an important factor affecting the yield of all biotechnological products because the capacity to produce a metabolite is bestowed by the genes of the organism. The bioindustrial production process is often dependent on the use of hyperproducing microbial strains: even with cheap raw materials, optimized medium and culture conditions, and efficient recovery processes, a production process cannot be made commercially viable and profitable until the yield of the final product by the producer organisms is naturally high. Moreover, the industrial production process is dependent on the availability of recombinant and mutant hyperproducers if good yields are lacking from the natural

producer strains. Even if high-yielding natural strains are available, the recombinant hyperproducers are always required, to economize further the production process and to obtain products with better commercially important properties. Besides the natural biosurfactant producer strains, a few mutant and recombinant varieties with enhanced biosurfactant production characteristics are reported in the literature. These mutant varieties were produced using various agents, for example, transposons[80], chemical mutagens such as N-methyl-N'nitro-N-nitrosoguanidine[81], radiation [82] or by selection on the basis of resistance to ionic detergents such as cetyl tri methyl ammonium bromide (CTAB) [83]. In addition to these mutant hyperproducing varieties, several recombinant strains producing biosurfactants in better yields and showing improved production properties have been developed in recent years (Table 3).

Table 3

Mutant and recombinant strains of microorganisms with enhanced biosurfactant yields and with improved product characteristics

Mutant and/or recombinant strain	Characteristic feature	Increased yield and/or improved production properties
Pseudomonas aeruginosa 59C7	Transposon Tn5-GM induced mutant of *Pseudomonas aeruginosa* PG201	2 times more production
Pseudomonas aeruginosa PTCC 1637	Random mutagenesis with N-methyl-N0-nitro-Nnitrosoguanidine	10 times more production
Bacillus licheniformis KGL11	Random mutagenesis with N-methyl-N0-nitro-Nnitrosoguanidine	12 times more production
Bacillus subtilis ATCC 55033	Random mutagenesis withN-methyl-N0-nitro-Nnitrosoguanidine	Approximately 4–6 times
Pseudomonas aeruginosa EBN-8	Gamma ray induced mutantof *Pseudomonas aeruginosa* S8	2–3 times more production
Acinetobacter calcoaceticus RAG-1	Mutant selection on basis of resistance to cationic detergent CTAB	2–3 times more production

1.13. PRODUCT RECOVERY OF BIOSURFACTANT

Even if optimum production is obtained using optimal media and culture conditions, the production process is still incomplete without an efficient and economical means for the recovery of the products. Thus, one important factor determining the feasibility of a production process on a commercial scale is the availability of suitable and economic recovery and downstream procedures. The choice of method for recovery of a particular biosurfactant depend on its ionic charge, solubility in water, whether the product is cell bound or extracellular and of course, the cost of recovery (Table 4). Several conventional methods for the recovery of biosurfactants, such as acid precipitation, solvent extraction, crystallization, ammonium sulfate precipitation and centrifugation, have been widely reported in the literature[33]. A few unconventional and interesting recovery methods have also been reported in recent years. A few examples of such biosurfactant recovery strategies include foam fractionation,[84] ultrafiltration[85], adsorption–desorption on polystyrene resins and ion exchange chromatography[86], and adsorption–desorption on wood-based activated carbon (WAC)[87]. One of the main advantages of these methods is their ability to operate in a continuous mode for recovering biosurfactants with high level of purity.

1.13.1. Solvents used for biosurfactant recovery

The solvents that are generally used for biosurfactant recovery, for example, acetone, methanol and chloroform, are toxic in nature and harmful to the environment. Cheap and less toxic solvents such as methyl tertiary-butyl ether (MTBE), ethyl acetate have been successfully used in recent years to recover biosurfactants produced by *Rhodococcus*[88]. These types of low cost, less toxic and highly available solvents can be used to cut the recovery expenses substantially and minimize the environmental hazards. Often, a single downstream processing technique is not enough for product recovery and purification. In such a case, a multi-step recovery strategy, using a sequence of concentration and purification steps, is much more effective[87]. In such a multistep recovery for biosurfactants, it will be possible to obtain the product at any required degree of purity.

Table 4

Physicochemical property-based biosurfactant recovery methods and their relative advantages

Downstream recovery procedure	Biosurfactant property responsible for separation	Instrument / apparatus / setup required	Advantages
Acid precipitation	Biosurfactants become insoluble at low pH values	No set-up required	Low cost, efficient in crude biosurfactant recovery
Organic solvent extraction	Biosurfactants are soluble in organic solvents due to the presence of hydrophobic end	No set-up required	Efficient in crude biosurfactant recovery and Partial purification, reusable nature
Ammonium sulfate precipitation	Salting-out of the polymeric or protein rich biosurfactant	No set-up required	Effective in isolation of certain type of polymeric biosurfactants
Centrifugation	Insoluble biosurfactants get precipitated because of centrifugal force	Centrifuge required	Reusable, effective in Crude biosurfactant recovery
Foam fractionation	Biosurfactants, due to surface activity, form and partition into foam	Specially designed bioreactors that facilitate foam recovery during fermentation	Useful in continuous recovery procedures, high purity of product
Membrane ultrafiltration	Biosurfactants form micelles above their critical micelle concentration (CMC), which are trapped by polymeric membranes	Ultrafiltration units with porous polymer membrane	Fast, one-step recovery, high level of purity
Adsorption on polystyrene resins	Biosurfactants are adsorbed on polymer resins and subsequently desorbed with organic solvents	Polystyrene resin packed in glass columns	Fast, one-step recovery, high level of purity, reusability
Adsorption on wood-activated	Biosurfactants are adsorbed on activated	No setup required, can	Highly pure biosurfactants,

carbon	carbon and can be desorbed using organic solvent	be added to culture broth, can also be packed in glass columns	cheaper, reusability, recovery from continuous culture
Ion-exchange chromatography	Charged biosurfactants are attached to ion-exchange resins and can be eluted with proper buffer	Ion-exchange resins packed in columns	High purity, reusability, fast recovery
Solvent extraction (using Methyl tertiary-butyl ether)	Biosurfactants dissolve in organic solvents owing to the hydrophobic ends in the molecule	No set-up required	Less toxic than conventional solvents, reusable, cheap

1.14. APPLICATIONS OF BIOSURFACTANTS

- Therapeutic and biomedical applications
- Pharmaceutical applications
- In food industry
- Environmental applications

1.14.1. THERAPEUTIC AND BIOMEDICAL APPLICATIONS

1.14.1.1. Antimicrobial activity

It is known that glycolipid biosurfactants show various biological activities reflecting their carbohydrate structure. Surfactants having solubilizing action show certain antimicrobial activates. In general the effect of biosurfactant on bacteria appears more markedly with gram-positive bacteria than gram-negative bacteria because of the different cell wall structures. Moreover, some glycolipid biosurfactants inhibit not only growth of microorganisms but also that of viruses.

1.14.1.1.1. Mannosyl erythritol lipids

The minimum inhibitory concentrations of Mannosyl erythritol lipids a glycolipid surfactant from *Candida antartica* against the bacteria are much smaller than those of sucrose monodecanoate and span 20[89].

1.14.1.1.2. Rhamnolipids

Rhamnolipids (RL-1 and RL-2) from *Pseudomonas aeruginosa* inhibit the growth of *Bacillus subtilis* in concentration of 10-35 mg/l. The lipids show antiphytoviral effects for the virus/host combination of tobacco mosaic virus/Nicotiana glutinosa and potato X virus/Nicotiana tobaccum. The lipids also exhibit zoosporicidal activity on species of three representative genera of zoosporic phytopathogen. Few data on the antifungal activity of biosurfactant are available so far. However, newly identified rhamnolipids from *Pseudomonas aeruginosa* AT 10, which are a mixture of seven homologues, show excellent growth inhibition activities not only for gram positive and negative bacteria but also excellent antifungal properties against *Aspergillus niger* (16 mg/mL) [90].

1.14.1.1.3. Trehalose lipids

Trehalose lipids (TL-1 and TL-2) show no growth inhibition against gram negative bacteria and yeasts. However, TL-1 inhibits the Conidia germination of fungus *Glomerella cingulata* at a concentration of 300mg/ml. On the other hand, succinoyl-trehalose lipids (STL-1 and STL-2) inhibit Harpes simplex virus and influenza virus at concentration of 11-33mg/L[91].

1.14.1.1.4. Sophorolipids

Sophorolipids (SL-1 and SL-2) inhibit the growth of *Bacillus subtilis, Staphylococcus epidermis* and *Streptococcus Faeciumat* at concentration of 6-29mg/L. SL-2 also inhibits the germination of Conidia of the fungus *Glomerella cingulata* at a concentration of 50mg/L. Different sophorolipids produced by *Candida apicola* inhibit the growth of not only gram-positive bacteria but also gram-negative bacteria such as *Escherichia coil* and *Serratia marcescens.*[92]

1.14.1.1.5. Lipopeptides

The lipopeptide iturin from *Bacillus subtilis* showed potent antifungal activity[93]. Inactivation of enveloped virus such as herpes and retrovirus was observed with 80 mM of surfactin[94].

1.14.1.2. Anticancer activity

1.14.1.2.1. Cell differentiation inducing activity

Recently, some microbial products have attracted attention as low-molecular bioprobes that can control a variety of mammalian cell functions. They are considered to participate in various intercellular molecular recognitions such as signal transduction, cell differentiation, cell immune response, etc.[95]

Glycolipid biosurfactants exhibits unique actions on mammalian cells in addition to antimicrobial and antiviral activities. Recent reports confirmed that Mannosyl erythritol-lipids (MEL-A and MEL-B), Succinoyl-trehalose lipids (STL-1 and STL-3), sophorolipids (SL) and polyol lipids (PL) have excellent growth inhibition and differentiation- inducing activites against human leukemia cells such as myelogenous leukemia cell K 562, promyelocytic leukemia cell HL 60 and basophilic-leukocyte KU812[96] MEL-A and MEL-B inhibit the growth of HL60 cells at concentration of 5-10mm, and induce drastically their morphological changes i.e. adhesions to the bottom of a cultivation flask.

Interestingly, the differentiation direction of these induced cells depends on the kind of biosurfactant with respect of HL60 cells, MEL-A, MEL-B and PL(1-mg/L) induce granulocytic differentiation, while SL(10mg/L), STL-1 (2.5μM) and STL-3 induces monocytic differentiation. In case of STL-3, the effect of the lipid on HL60 cells depend on its hydrophobic structures.[97] More significantly, all of these six glycolipid biosurfactants inhibit the activity of phospholipids and Ca^{2+}-dependent protein Kinase C of HL60 cells. MEL-A also inhibits the Serine/ Threonine phosphorylation of 30Kda protein in HL-60 cells and the tyrosine phosphorylation of 55-, 65-, 95-, 135- Kda proteins in K 562 cells. Therefore, MEL appears to directly affect intracellular signal transduction through phosphate cascade systems.

MEL-A has been recently demonstrated to inhibit the growth of mouse melanoma B16 cells in a dose-dependent manner. Exposure of B16 cells to the lipid causes the condensation of chromatin, DNA fragmentation and sub-G1 arrest, all of which are hall marks of cells that are undergoing apoptosis. The lipid treatment also enhances expression of PRC α-suggesting that the lipid triggers the differentiation of the cells through a signal pathway that involves PKCα. In addition, exposure of PC12 cells to mannosylerythritol enhanced the activity of acetylcholine esterase and interrupted the cell cycle at the G1 phase, with resulting outgrowth of neuritis and partial cellular differentiation[98]. This suggests that mannosylerythritol induces neuronal differentiation in PC12 cells and provides the groundwork for the use of microbial extracellular glycolipids as novel reagents for the treatment of cancer cells.

Another report suggested that the cytotoxic effects of the sophorolipid on cancer cells of H7402, A549, HL60 and K562 were investigated by MTT assay. The results showed a dose-dependent inhibition ratio on cell viability according to the drug concentration <62.5g/ml. These findings suggested that the sophorolipid produced by *Wickerhamiella domercqiae* have anticancer activity[99].

It is very surprising that some glycolipid biosurfactant show certain activities against various organisms ranging from prokaryote to mammalian. The complicated but naturally selected structures of glycolipid biosurfactants allow them to display versatile performance over what would be attained by conventional chemical surfactants. Therefore, the functional development of biosurfactants should also be carried out from a view point of biochemistry and molecular biology.

1.14.1.3. Affinity binding to human immunoglobulin G

Immunoglobulin G (IgG) which is the most dominant and essential immunoglobulin in mammalians is widely used in immunodiagnostics and therapeutic applications. Because of its high affinity, protein-A is the usual choice for immuno affinity chromatography of IgG . Chromatography using protein A, however, has two major drawbacks the high cost of the ligand protein and the highly acidic conditions needed to elute IgG. On the other hand, some of the glycolipids such as gangliosides exhibit high affinity for immunoglobulins and thus are focused on a few affinity ligand for IgG. The possibility of developing these membrane glycolipids into new ligands, however is far from straight forward due to their limited amounts and heterogeneity.

In an ELISA assay, MEL-A showed nearly the same binding affinity towards the protein as that of bovine ganglioside. Interestingly, MEL-A non covalently attached onto poly (2-hydroxyethyl methacrylate) beads and exhibited a significant binding constant of $1.43x10^{6}$ (M^{-1}) for the protein which is approximately four times greater that that of protein- A previously reported.

1.14.1.4. Immuno modulatory action

Sophorolipids are promising modulators of the immune response. They have previously demonstrated that sophorolipids (1) decreased sepsis related mortality at 36 h *in vivo* in a rat model of septic peritonitis by modulation of nitric oxide, adhesion molecules and cytokine production and (2) decreased IgE production *in vitro* in U266 cells possibly through affecting plasma cell activity. The result shows sophorolipids decrease IgE production in U266 cells by downregulating important genes involved in IgE pathobiology in a synergistic manner. These data continue to support the utility of sophorolipids as an anti-inflammatory agent and novel potential therapy in diseases of altered IgE regulation[100].

1.14.1.5. Anti-human immunodeficiency virus and sperm-immobilizing activity

The increased incidence of human immunodeficiency virus (HIV)/AIDS disease in women aged 15 to 49 years has identified the urgent need for a female-controlled, efficacious, and safe vaginal topical microbicide. To meet this challenge, sophorolipid (SL) produced by *Candida bombicola* and its structural analogs have been studied in this report for their spermicidal, anti-HIV, and cytotoxic activities[101]. The sophorolipid diacetate ethyl ester derivative is the most potent spermicidal and virucidal agent of the series of sophorolipids studied. Its virucidal activity against HIV and sperm-immobilizing activity against human semen are similar to those of nonoxynol-9. However, it also induced enough vaginal cell toxicity to raise concerns about its applicability for long-term microbicidal contraception.

1.14.1.6. Application of biosurfactants to gene delivery carriers

1.14.1.6.1. Dramatic increase in efficiency of gene transfection

Gene transfection across cell membranes is a fundamental technology for molecular and cell biology and for clinical gene therapy of cancer. Although the most efficient methods for gene transfection involve the use of viral vectors, there are still arguments about risks in regard to propagation and immunogenicity. A variety of non-viral gene-delivery systems has been investigated, and the system using liposomes are much convenient and safer than viral system. Cationic liposomes with cationic cholesterol or polymer derivatives have been widely used due to their high transfection efficiency and low toxicity. Some success in getting DNA and RNA in the cells has been achieved with the cationic liposomes. However gene delivery mediated by liposomes still remains an efficient process.

Recently MEL-A has been demonstrated to dramatically increase in the efficiency of gene transfection mediated by the liposomes with a cationic cholesterol derivatives.[102] Among the cationic liposomes tested, the liposomes bearing cholesteryl 1-3- β-carboxyaminodoethylene- N-hydroxyethylamine and

MEL-A showed the best efficiency for delivery of plasmids encoding luciferase (pGL3) into the target cells (NIH3T3, COS-7 and HeLa.).

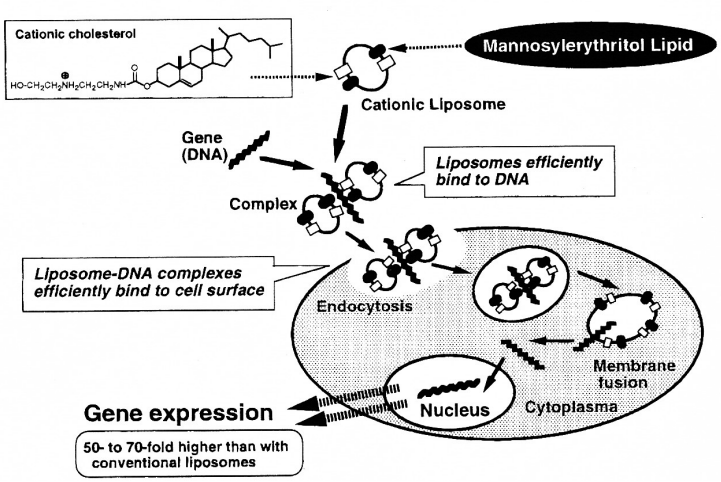


Fig. 5: Scheme of gene delivery into mammalian cells using cationic liposomes including mannosylerythritol lipids

1.14.1.7. Antiadhesive agents in biomedicine

Pre-treatment of silicone rubber with *S. thermophilus* surfactant inhibited by 85% the adhesion of *Candida albicans*[103] whereas surfactants from *Lactobacillus fermentum* and *Lactobacillus acidophilus* adsorbed on glass, reduced by 77% the number of adhering uropathogenic cells of *Enterococcus faecalis*. Lately, the biosurfactant from *L. fermentum* was reported to inhibit *Staphylococcus aureus* infection and adherence to surgical implants[104]. Surfactin decreased the amount of biofilm formation by *Salmonella typhimurium, Salmonella enterica, E. coli* and *Proteus mirabilis* in PVC plates and vinyl urethral catheters[105]. The use of biosurfactants, which disrupts biofilms and reduce adhesion, in combination with antibiotics could represent a novel antimicrobial strategy, once antibiotics are in general less effective against biofilms than planktonic cells; the disruption of biofilm by biosurfactant can facilitate the antibiotic access to the cells[106].

1.14.2. PHARMACEUTICAL APPLICATIONS

- Active surfactants in pharmaceutical formulations (orals and parenterals).
- Preparations of germicidal formulations.
- In cosmetics.
- Foam booster for bubble baths, shampoo, toothpastes, shaving cream / foam/ gas, and other soap based products.
- Emulsifier for skin creams, conditioners, degreasers and cleansers.
- Anti dandruff agents.
- Preparation of creams, ointments, lotions and emulsions.

1.14.3. IN FOOD INDUSTRY

1.14.3.1. Food formulation ingredients

Apart from their obvious role as agents that decrease surface and interfacial tension, thus promoting the formation and stabilization of emulsions, surfactants can have several other functions in food. For example to control the agglomeration of fat globules, stabilize aerated systems, improve texture and shelf-life of starch-containing products, modify rheological properties of wheat dough and improve consistency and texture of fat-based products[107]. In bakery and ice cream formulations biosurfactants act controlling consistency, retarding staling and solubilizing flavor oils; they are also utilized as fat stabilizer and antispattering agent during cooking of oil and fats. An improvement of dough stability, texture, volume and conservation of bakery products was obtained by the addition of rhamnolipid surfactants[108].

1.14.3.2. Antiadhesive agents in food industry

A biofilm is described as a group of bacteria that have colonized a surface. The biofilm not only includes the bacteria, but it also describes all of the extracellular material produced at the surface and any material trapped within the resulting matrix. The first step on biofilm establishment is bacterial adherence which is affected by factors including microorganism species, hydrophobicity of surface and electrical charges involved, environmental conditions and ability of microorganisms to produce extracellular polymers that help cells to anchor to surfaces. Bacterial biofilms present in food industry surfaces are potential sources

of contamination, which may lead to food spoilage and disease transmission. Thus controlling the adherence of microorganisms to food contact surfaces is an essential step in providing safe and quality products to consumers[109].The involvement of biosurfactants in microbial adhesion and detachment from surfaces has been investigated. The bioconditioning of surfaces through the use of microbial surfactants have been suggested as a new strategy to reduce adhesion.

1.14.4. ENVIRONMENTAL APPLICATIONS[110]

Hydrophobic pollutants present in petroleum hydrocarbons, soil and water environment require solubilization before being degraded by microbial cells. Mineralization is governed by desorption of hydrocarbons from soil. Surfactants can increase the surface area of hydrophobic materials, such as pesticides in soil and water environment, thereby increasing their water solubility. Hence the presence surfactants may increase microbial degradation of pollutants .Use of biosurfactants for degradation of pesticides in soil and water environment has gained importance recently.

A number of industrial applications of biosurfactants have been envisaged. They are very selective, required in small amounts, effective under broad ranges of conditions and eco friendly especially in the safeguarding the coastal areas from additional damage caused by synthetic chemicals.

1.14.4.1. Rhamnolipids

1.14.4.1.1. Effect of Rhamnolipid on Contaminant Biodegradation

a.) Petroleum hydrocarbons:

The various components of petroleum hydrocarbons are alkanes, cycloalkanes, aromatics, polycyclic aromatic hydrocarbons, asphaltanes. Various studies have examined the effect of rhamnolipids on biodegradation of organic contaminants with mixed results. A recent review indicated that rhamnolipid addition can enhance biodegradation of hexadecane, octadecane, n-paraffin and phenanthrene in liquid systems.

Two mechanisms for enhanced biodegradation are possible, [111]

i) Enhanced solubility of the substrate for the microbial cells.

ii) Interaction with the cell surface, which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily.

A concentration of 300 mg/l of rhamnolipids increased the mineralization of octadecane to 20% from 5% for the controls.

b) Polynuclear aromatic hydrocarbons (PAHs):

Polycyclic or polynuclear aromatic hydrocarbons (PAHs) are components of creosti and are produced during petroleum refining, coke production and wood preservation. Many are suspected to be carcinogens.

Researchers have compared[112] the solubilisation of the PAH, naphthalene, by a rhamnolipid, sodium dodecyl sulpfate (SDS), Trition X-100. The biosurfactant increased the solubility of naphathalene by 30 times.

c) Chlorinated hydrocarbons:

DDT, 2, 4-D and 2, 45-T, plasticisers, pentachlorophenol, polychlorinated biphenyls, among others are examples of halogenated aromatic compounds and highest ranking pesticide used in India. They are carcinogen and their solubility and toxicity are causes of great concern for the environment and public health.

Using 4g/l of biosurfactant, 4, 4' chlorobiphenyl was mineralized by 213 times more than the control. Pesticides are another group of contaminants that have been studied. A study compared the ability of the rhamnolipid mixture to solublize the pesticides with the synthetic surfactant Trition X-100. The synthetic surfactant was able to solubalize approximately twice as much of all pesticides as the rhamnolipid.

1.14.4.1.2. Exsitu Washing Studies by Rhamnolipids

a) Oil

Besides studies on biodegradation, rhamnolipid surfactants have been tested to enhance the release of low solubility compounds from soil and other solids. Removal efficiency varied according to contact time and biosurfactant concentration.[113]

Rhamnolipids from *Pseudomonas aeruginosa* UG2 were able to effectively remove a hydrocarbon mixture from a sandy loam soil and that the degree of removal was dependent on the type of hydrocarbon removed and the concentration of surfactant used. The same strain could remove at a concentration of 5g/l, approximately 10% more hydrocarbons from a sandy loam soil than a slit loam soil and that sodium dodecyl sulfate (SDS) was less effective than the biosurfactants in removing hydrocarbons.

b) Heavy metals:

Due to the anionic nature of rhamnolipids, they are able to remove metals from soil and ions such as cadmium, copper, lanthanum, lead and zinc due to their complexation ability[114], more information is required to establish the nature of the biosurfactant-metal complexes. Due to the foaming property of biosurfactant, metal-biosurfactant complexes can be removed by addition of air to cause foaming and then the biosurfactant can be recycled through precipitation by reducing the pH to 2.

1.14.4.1.3. Remediation of oil-contaminated water by rhamnolipids

a) Hydrocarbon degradation in aquatic environment

When oil is spilled in aquatic environment is a major problem that can destroy coastlines. The lighter hydrocarbon components volatilize while the polar hydrocarbon components dissolve in water. However, because of low solubility (< 1 ppm) of oil, most of the oil components will remain on the water surface. The primary means of hydrocarbon removal are photooxidation, evaporation, and microbial degradation. Since C*x*H*y*-degrading organisms are present in seawater, biodegradation may be one of the most efficient methods of removing pollutants. Surfactants enhance degradation by dispersing and emulsifying hydrocarbons.

Microorganisms that are able to degrade C*x*H*y* have been isolated from aquatic environment. These microorganisms which exhibit emulsifying activity as well as the soil microorganisms which produced surfactants may be useful in aquatic environment.

Another recent development is the feasibility of biosurfactants for, a solution of 2% rhamnolipids diluted in saline water and applied at a dispersant to oil ratio (DOR) of 1:2, immediately dispersed 65% of a crude oil. Co-addition of 60% ethanol and 32% octanol with 8% rhamnolipids applied at a DOR of 1:8 improved dispersion to 82%.

1.14.4.2. Sophorolipids

1.14.4.2.1. Effect on biodegradation and contaminated removal by sophorolipids

A crude preparation of biosurfactants from *Candida bombicola* was able to partially solubalize lignite coal.[115] Metal contaminated soils and sediments have been treated with crude sophorolipids resulted in the removal of heavy metals.

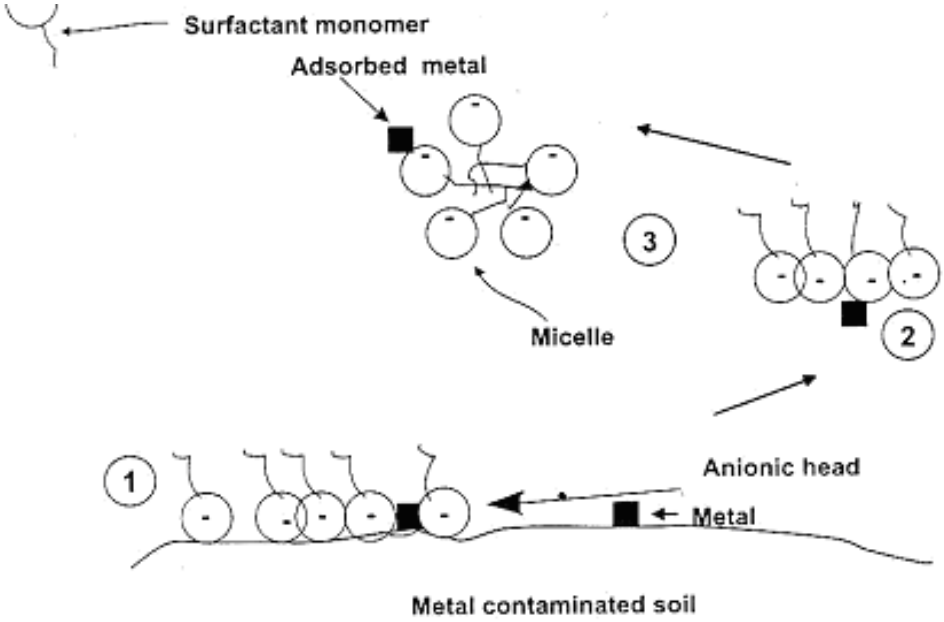
The concentration of phenanthrene (initial concentration of 80 mg/l) within 36 h decreased to 0.5 mg/l in the presence of 500 mg/l of the surfactant compared to 2.3 mg/l without surfactant in a 10% soil suspension. In addition, toxicity of the sophorolipid was low for concentration upto 1 g/l. The CMC of the sophorolipid in water was 4 mg/l, but this increased to 10 mg/l in the presence of 10% soil suspension indicating adsorption of the surfactant onto the soil.

These experiments have indicated that the sophorolipids enhance biodegradation of the phenanthrene through enhanced solubalization.

1.14.4.3. Surfactin

1.14.4.3.1. Ex situ washing studies by surfactin

Surfactin is another biosurfactant that has been evaluated for environmental applications. It was indicated that surfactin was able to remove the metals by sorption at the soil interface and metal complexation, followed by desorption of the metal through lowering of soil-water interfacial tension and fluid forces, and finally complexation of the metal with the micelles. (Fig 6)



1. Accumulation of surfactant as hemimicelles or admicelles at soil interface
2. Removal of metal by lowering of interfacial tension and electrostatic attraction
3. Incorporation of metal into micelle

Fig. 6: Potential mechanism for metal removal by a biosurfactant, surfactin

1.14.4.3.2. Remediation of heavy-metal contaminated water by surfactin

Using a technique called micellar enhanced ultrafiltration, Mulligan[116] et al. (1999b) studied the removal of various concentrations of metals from water by various concentrations of surfactin by a 50,000 Da molecular weight cutoff ultrafiltration membrane. (Fig. 7) Cadmium and zinc rejection ratios were superior (close to 100%) at pH values of 8.3 and 11 while copper rejection ratios were highest at pH 6.7 (about 85%). The addition of 0.4% oil as a co-contaminant decreased slightly the retention of the metals by the membrane. The ultra filtration membranes also indicated that metals became associated with the surfactin micelles as the metals remained in the retentate and did not pass through into the permeate.

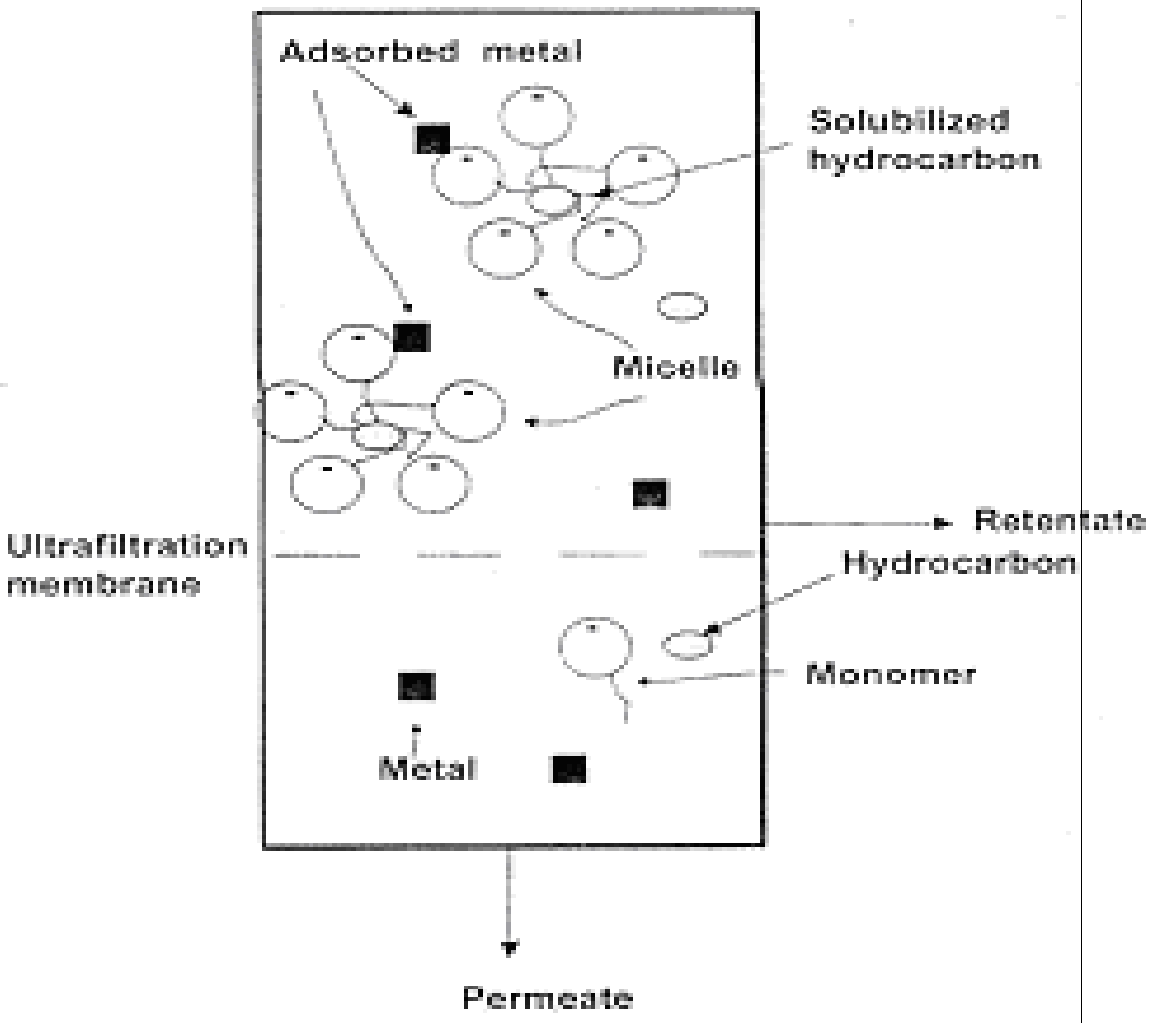


Fig. 7: Schematic diagram of ultrafilteration of surfactant- contaminant (hydrocarbon and metal) complexes

Table 5

Industrial applications of biosurfactants

Industry	Application	Role of biosurfactants
Petroleum	Enhanced oil recovery	Improving oil drainage into well bore; stimulating release of oil entrapped by capillaries; wetting of solid surfaces; reduction of oil viscosity and oil pour point; lowering of interfacial tension; dissolving of oil
	De-emulsification	De-emulsification of oil emulsions; oil solubilization; viscosity reduction, wetting agent
Environmental	Bioremediation	Emulsification of hydrocarbons; lowering of interfacial tension; metal sequestration
	Soil remediation and flushing	Emulsification through adherence to hydrocarbons; dispersion; foaming agent; detergent; soil flushing
Food	Emulsification and de-emulsification	Emulsifier; solubilizer; demulsifier; suspension, wetting, foaming, defoaming, thickener, lubricating agent
	Functional ingredient	Interaction with lipids, proteins and carbohydrates, protecting agent
Biological	Microbiological	Physiological behaviour such as cell mobility, cell communication, nutrient accession, cell–cell competition, plant and animal pathogenesis
	Pharmaceuticals and therapeutics	Antibacterial, antifungal, antiviral agents; adhesive agents; immunomodulatory molecules; vaccines; gene therapy
Agricultural	Biocontrol	Facilitation of biocontrol mechanisms of microbes such as parasitism, antibiosis, competition, induced systemic resistance and hypovirulence
Bioprocessing	Downstream processing	Biocatalysis in aqueous two-phase systems and microemulsions; biotransformations; recovery of intracellular products; enhanced production of extracellular enzymes and fermentation products
Cosmetic	Health and beauty products	Emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agent, mediators of enzyme action

1.15. ABOUT SOPHOROLIPIDS

Sophorolipids are glycolipid type of biosurfactants which consists of a dimeric carbohydrate sophorose linked to long-chain hydroxy carboxylic acids by glycosidic linkage. Generally sophorolipids occurs as mixture of macrolactones and free acid form. Yeast has been shown to be potent producers of sophorolipids as an extra cellular biosurfactant. *Torulopsis bombicola* produces a sophorolipid like biosurfactant during alkane fermentation[12] while *Torulopsis petrophilum* and *Torulopsis apicola* are major producers *o*f sophorolipids[13, 14].Natural mixtures of sophorolipids, pure isolates of individual components, and their derivatives have shown significant promise as therapeutic agents. They possess spermicidal and virucidal properties[101]. They act as immunomodulators in the treatment of septic shock by cytokine down regulation[100] .They exhibit anti cancer activity[99] and are used for the treatment of skin diseases. Although sophorolipids are usable in a broad spectrum of industrial areas such as cosmetics, soil decontamination, food, pharmaceutics etc and despite the fact that a number of patents are available regarding their applications, a real breakthrough of these compounds has not yet taken place.

It has been shown that the lactone form of the sophorolipid is necessary, or at least preferable, for many of these applications [117].It is fortunate that in the mixtures of sophorolipids obtained from the yeasts, the various lactonic forms usually represent the largest fraction of the product [13,118,119]. However, Hu and Ju point out that it is still necessary to separate the lactonic forms from the acidic forms for maximum effectiveness of these compounds. They concluded that the previous attempts to isolate the lactones have all involved expensive procedures. Furthermore, depending on cultivation conditions and carbon sources[120] up to 12 different sophorolipids [118] were found. These include variations of both the acidic and lactonic forms, which exhibit differences in the length of the fatty acid component, the presence of unsaturations in the fatty acid chain and the degree of acetylation. The sophorolipids are usually obtained as mixtures of all of the above compounds [119,121].These mixtures are typically brown oils, which are viscous and denser than water. Although sophorolipid production occurs in the presence of a

simple carbohydrate substrate, it has been established that the highest yield of sophorolipids are obtained when an additional, hydrophobic substrate is provided[121, 122.] These authors suggested that the water-soluble substrate is used primarily for cellular metabolism and for synthesis of the hydrophilic sophorolipid moiety, while the lipophilic substrate is used exclusively for production of the hydroxy-carboxylic acid moiety.

2. SCOPE AND PLAN OF WORK

The enormous market demand for surfactants is currently met by numerous synthetic, mainly petroleum-based chemical surfactants. These compounds are usually toxic to the environment and non-biodegradable. They may bio-accumulate and their production, processes and by-products can be environmentally hazardous. Tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternatives to chemical surfactants[1]. Biosurfactants have become recently an important product of biotechnology for industrial and medical applications. The reason for their popularity, as high value microbial products, is primarily in their specific action, higher biodegradability and low toxicity, structural diversity, greater environmental compatibility, stable activity at extremes of pH, salinity and temperature, relative ease of preparation and widespread applicability[33,123.]. They can be used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredients and detergents in various industrial sectors such as, Petroleum and Petrochemicals, Organic Chemicals, Foods and Beverages, Cosmetics and Pharmaceuticals, Mining and Metallurgy, Agrochemicals and Fertilizers, Environmental Control and Management, importance in the fields of enhanced oil recovery, environmental bioremediation, food processing[3]. They have also been found to possess several properties of therapeutic and biomedical importance: they have antibacterial, antifungal and antiviral properties; they inhibit fibrin clot formation; immunomodulatory molecules, anti-human immunodeficiency virus and sperm-immobilizing activity, they have anti-adhesive action against several pathogenic microorganisms, they can be used as agents for respiratory failure and agents for stimulating skin fibroblast metabolism etc[3, 124,125].

The interest in the production of biosurfactant has steadily increased during past decade. The improvement in the production technology of biosurfactants has already enabled 10-20 fold increase productivity. Various studies reported on the production of biosurfactant from *Candida* species in large quantities. This area of biosurfactant research is still in its infancy. In the current study, various *Candida* species was used as test organism for the production of sophorolipids.

The aim of study is to isolate, optimize, characterize and identify the active components of the biosurfactants from various *Candida* species. And also to evaluate different media for the optimum production of biosurfactants,

The aim of current study in the following stages:

Stage1: Screening for biosurfactant producing microorganisms.

Stage2: Cultural and biochemical characterization of biosurfactant producing *Candida* species.

Stage3: Optimization of medium composition.

Stage4: Optimization of cultural conditions.

Stage5: Fermentative production of sophorolipids by using optimized medium.

Stage6: Extraction and partial purification of sophorolipids.

Stage7: Characterization of sophorolipids.

Stage8: Analysis of sophorolipids.

Stage9: Evaluation of possible activities of sophorolipids

3. LITERATURE REVIEW

- It was only in the 1950's that microbial polymers were investigated and latter commercially produced. Microbial surfactants have received much attention because of their potential applications in various fields like biomedicine, pharmaceuticals, food industries and oil recovery plants.
- From 1960's considerable amount of work on production and characterization of microbial surfactants have been performed. Some of the recent works that they carried are reviewed below.
- Cirigliano and Carman 1984 [45] produced a bioemulsifier from *Candida lipolytica*. The yeast *Candida lipolytica* produced an inducible extracellular emulsification activity when it was grown with a number of water-immiscible carbon substrates. Negligible emulsification activity was produced by this yeast when it was grown with glucose as the carbon substrate. In hexadecane-supplemented cultures, emulsification activity was first detected after 36 h of growth, with maximum production after 130 h.. This emulsifier, which they named liposan, was primarily composed of carbohydrate. Maximum emulsification activity was obtained when the ratio of hexadecane to liposan was 50:1. Maximum emulsification activity was obtained from pH 2 to 5. Liposan was heat stable to temperatures up to 70 ^{0} C, with a 60% loss in activity after heating for 1 h at 100 ^{0} C. Liposan effected stable oil-in-water emulsions with a variety of hydrocarbons.
- Singh and Desai 1989 [43] studied hydrocarbon emulsification by *Candida tropicalis* and *Debaryomyces polymorphus.* Potentiality of *Candida tropicalis* and *Debaryomyces polymorphus*, to produce surface active compounds (bioemulsifiers/biosurfactants) during shake cultivation on hexadecane and oily waste was studied. Better emulsification activity, specific towards aromatic hydrocarbons, was observed with *Candida tropicalis* culture broth. Emulsification activity of culture broth was quite stable and was unaffected by change in pH and by increasing the concentration of NaCl up to 5%. The

activity was marginally affected by heating in boiling water bath for 15 min, but inhibited to the tune of 90% by 0.3% $CaCl_2$. The isolated bioemulsifying factor contained 40, 22 and 17.5% lipid, protein and carbohydrate, respectively.

- Davila *et al.,* 1993 [126] used high performance liquid chromatography for the characterization of sophorolipids by using gradient elution with a water - acetonitrile mixture on a reversed phase (C18) column and evaporative light scattering detection, resolution of all the important individual sophorolipids present in the fermentation product was achieved. The chemical conversion of the fermented sophorolipids was done and they were separated and identified by GC-MS.

- Deshpande and Daniels 1995 [65] had been studied evaluation of sophorolipid biosurfactant production by *Candida bombicola* using animal fat. The meat processing industry is seeking new applications for abundantly available, inexpensive animal fats. Sophorolipid production by *Candida bombicola* was studied as a model for fat utilization for biosurfactant production. Inoculum medium components (glucose, animal fat, ammonium sulfate, corn steep liquor, urea and potassium phosphate) were optimized for rapid growth. Similar components were chosen to design a production medium for sophorolipid production. Maximum growth was obtained at 30°C but greater sophorolipid production occurred at 27°C. In a pH controlled fermenter (maintained at pH 3"4 after the first 24 h) 120 g 1^{-1} of sophorolipid was obtained in 68 h. Silica gel column chromatography of the extracted sophorolipid revealed the presence of atleast six components, including a major component comprising 45% of the total sophorolipids. Cells at the end of fermentation contained 37% protein and 14% lipids. The animal fat used was white choice grease, from a hog processing plant as a corbon source.

- Healy *et al.* 1996 [7] has reviewed microbial production of biosurfactants. Here they suggested biosurfactants are those chemicals which are produced by microorganisms but which have both clearly defined hydrophilic and hydrophobic groups. They occur in nature in bacteria, yeasts, and fungi. The

four main types of biosurfactant are: (1) glycolipids, (2) phospholipids, (3) lipoproteins or lipopeptides, (4) polymeric. By growth of the bacterium *Pseudomonas fluorescens* (NCIM B11712) on virgin olive oil, production of a glycolipid in the form of a rhamnolipid is thought to have taken place. This biosurfactant group is based on the rhamnose structure, which is a methyl pentose monosaccharide. However, rhamnolipids can also be based on the disaccharide by condensing two moles of rhamnose together. The link to the hydrophobic group is by way of an acetal group; however, the 'lipid' part of the molecule contains ester and carboxyl groups. Biosurfactants over the years have found a great many uses in industry, for example, (1) oil recovery, (2) oil spill clean-up, (3) textiles, (4) pharmaceuticals, (5) cosmetics.

- Casas *et al.* 1997 [127] has studied optimization of a synthetic medium for *Candida bombicola* growth using factorial design of experiments. The yeast *Candida bombicola* produces a mixture of sophorolipids when it grows on glucose and/or oil substrates. Maximum sophorolipid production has been achieved using a resting cell process. A good knowledge of growth conditions and medium formulation are necessary to obtain enough biomass in the sophorolipid production process. Two facts permit the study of yeast growth and sophorolipid production separately. These compounds are nongrowth-associated products. Two carbon sources for the sophorolipid synthesis are necessary. In this work, an optimization of a synthetic medium for *Candida bombicola* growth has been achieved by factorial design and analysis of experiments.

- Sarubbo *et al.* 1999 [128] had studied the use of babassu oil as substrate to produce bioemulsifiers by *Candida lipolytica*. *Candida lipolytica* IA 1055 produced an extracellular emulsifier when using babassu oil as its sole carbon source during batch and fed batch fermentations at 27^0. Emulsification activity was detected after 60 h of growth in all conditions studied. The bioemulsifier was isolated after 144 h of fermentation from the best condition studied. The biopolymer seems to be a polysaccharide-protein-lipid complex.

- Rosenberg and Ron 1999 [8] had classified biosurfactants as high- and low-molecular-mass microbial surfactants. Microorganisms synthesize a wide variety of high- and low-molecular-mass bioemulsifiers. The low-molecular-mass bioemulsifiers are generally glycolipids, such as trehalose lipids, sophorolipids and rhamnolipids, or lipopeptides, such as surfactin, gramicidin S and polymyxin. The high-molecular-mass bioemulsifiers are amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers. The low-molecular-mass bioemulsifiers lower surface and interfacial tensions, whereas the higher-molecular-mass bioemulsifiers are more effective at stabilizing oil-in-water emulsions. Bioemulsifiers have several important advantages over chemical surfactants, which should allow them to become prominent in industrial and environmental applications.

- Rau *et al.* 2001 [120] used renewable sources such as rapeseed oil or oleic acid, combined with glucose for the production of sophorolipids by using *Candida bombicola* ATCC 22214. High yields >300 gL^{-1} and increased productivities of 57 $gL^{-1}d^{-1}$ (fed-batch) and 76 gL^{-1} d^{-1} (Continuous mode), respectively, were obtained by using optimized cultivation conditions. The acidic sophorolipid was used for further modification after alkaline hydrolysis. A novel glycolipid was synthesized by using the enzyme naringinase (EC 3.2.1.40). A (ω-1) hydroxy fatty acid, commercially not yet available and difficult to prepare by organic synthesis, was released by acidic hydrolysis, purified and is proposed as a precursor for plastics and flavours.

- Daniel and Andrew 2001 [129] were studied microorganism selection and biosurfactant production in a continuously and periodically operated bioslurry reactor. A continuous-flow reactor (CSTR) and a soil slurry-sequencing batch reactor (SS-SBR) were maintained in 8 L vessels for 180 d to treat a soil contaminated with diesel fuel (DF). Concentrations of *Candida tropicalis, Brevibacterium casei, Flavobacterium aquatile Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* were determined using fatty acid methyl ester

(FAME) analysis. The SS-SBR encouraged the growth of biosurfactant-producing species relative to the CSTR. Counts of biosurfactant-producing species (*C. tropicalis, P. aeruginosa, P. fluorescens*) relative to total microbial counts were 88% in the SS-SBR and 23% in the CSTR. No biosurfactant production was observed in the CSTR. However, considerable foaming occurred in the SS-SBR. These results show that bioslurry reactor operation can be manipulated to control overall reactor performance.

- Hu and Ju 2001[130] studied various experimental methods for purifying lactonic sophorolipids via crystallization. They found that commonly used solvent, ethanol had much higher solubility for the lactonic sophorolipids than for the acid sophorolipids. Both phthalate and phosphate buffers were found more suitable for purifying lactonic sophorolipids than ethanol. A practical and effective method for purifying lactonic sophorolipids to about 99% purity using phosphate buffers was developed.

- Nunez *et al.* 2001[131] developed a reversed phase high performance liquid chromatographic method combined with atmospheric pressure chemical ionization mass detection (LC / APCI - MS) for the separation and analysis of sophorolipids produced by *Candida bombicola* when grown on fatty acid mixtures. Using this method it was found that the incorporation of palmitic, linolenic and linoeic acids in to the sophorolipid structure was dependent on the initial fatty acid content of these acids, whereas the incorporation of oleic acid was independent of its initial content in the mixture. He found that the combination of APCI-MS with reverse phase HPLC is an excellent tool for the analysis and identification of sophorolipids.

- Makkar and Cameotra 2002 [132] had demonstrated an update on the use of unconventional substrates for biosurfactant production and their new applications**.** Biosurfactants are valuable microbial amphiphilic molecules with effective surface-active and biological properties applicable to several industries and processes. Microbes synthesize them, especially during growth on water-immiscible substrates, providing an alternative to chemically prepared

conventional surfactants. Because of their structural diversity (i.e., glycolipids, lipopeptides, fatty acids, etc.), low toxicity, and biodegradability, these molecules could be widely used in cosmetic, pharmaceutical, and food processes as emulsifiers, humectants, preservatives, and detergents. Moreover, they are ecologically safe and can be applied in bioremediation and waste treatments. They can be produced from various substrates, mainly renewable resources such as vegetable oils, distillery and dairy wastes, which are economical but have not been reported in detail. In this review, they reports advances made in using renewable substrates for biosurfactant production and their newer applications.

- Muthusamy 2003 [133] reviewed the application of biosurfactants in industries. This review included the potential applications of microbial surface active compounds, based on their broad range of functional properties that include emulsification, phase separation – wetting, foaming, solubilization, corrosion – inhibition and viscosity reduction. There are therefore, many areas of industrial applications where chemical surfactants could be replaced by biosurfactants in fields as diverse as agriculture, food and beverage industries, cleaning, cosmetics, pharmaceutical industries and last but not the least, petroleum and petrochemical industries

- Cavelero and Cooper 2003 [134] studied the effect of medium composition on the structure and physical state of sophorolipids produced by *Candida bombicola* ATCC 22214 using a variety of lipophilic carbon substrates. Most of the hydrocarbon and carboxylic acid substrates resulted in a mixture of sophorolipids consisting of free acids and the more desirable lactones. The ratio of diacylated lactone to free acid in these mixtures was maximum when they were produced using hexadecane and heptadecane. All the other lipophilic substrates resulted in significant amounts of free acids being produced. These lactone products were unique in that they were precipitated as crystals, which were easily separated from the culture medium. All of the other products were isolated as oil as is usually reported in the literature. Finally, the amounts of

these crystals recovered were significantly higher than those observed for any of the oily products.

- Cameotra *et al.* 2004 [3] reviewed recent applications of biosurfactants as biological and immunological molecules. They described, in addition to the classical application as emulsifiers of hydrocarbons, biosurfactants can be used in environmental protection, crude-oil recovery, and food-processing industries and in various fields of biomedicine over chemical surfactants due to their lower toxicity and higher biodegradability, and are likely to become molecules of the future in areas such as biomedicine and therapeutics. They discussed the role and applications of biosurfactants mainly glycolipids and lipopeptides focusing on medicinal and therapeutic perspectives such as anti microbial and anti fungal agents, cell differentiation and cytotoxic activities.

- Youssef *et al.* 2004[21] had compared some of the methods to detect biosurfactant production by diverse microorganisms.Three methods to detect biosurfactant production, drop collapse, oil spreading,and blood agar lysis, were compared for their ease of use and reliability in relation to the ability of the cultures to reduce surface tension. The three methods were used to test for biosurfactant production in 205 environmental strains with different phylogenetic affiliations. Surface tension of select strains that gave conflicting results with the above three methods was also measured. The use of the drop collapse method as a primary method to detect biosurfactant producers, followed by the determination of the biosurfactant concentration using the oil spreading technique, constitutes a quick and easy protocol to screen and quantify biosurfactant production. The large number of false negatives and positives obtained with the blood agar lysis method and its poor correlation to surface tension (rs = -0.15) demonstrated that it is not a reliable method to detect biosurfactant production.

- Zhang *et al.* 2004[135] produced and studied synthesis and interfacial properties of sophorolipid derivatives. Biosurfactants made by fermentation from renewable resources provide "environmental friendly" processes and products. A natural sophorolipid mixture was produced by the yeast *Candida bombicola* when cultured on glucose and oleic acid. The sophorolipid mixture was chemically modified to form the corresponding sophorolipid alkyl (methyl, ethyl, propyl, and butyl) esters by reaction with the corresponding sodium alkoxides. Interfacial properties of these surfactants, such as surface tension reduction, aggregation, and adsorption, were systematically studied. It was found that the critical micelle concentration of sophorolipid esters decreases to about 1/2 per additional one CH_2 group to the alkyl ester moiety. Interestingly, these surfactants were found to adsorb strongly on alumina but weakly on silica. They have properties that make them attractive candidates for uses in detergents, cosmetics, soil remediation, and enhanced oil recovery.

- Maneerat 2005[136] reviewed production of biosurfactants using substrates from renewable-resources. This review suggested that surface-active compounds commonly used in industries are chemically synthesized. However, biosurfactants have been paid increasing attention to replace the synthetic surfactants owing to their advantages such as biodegradability and low toxicity. Nowadays, the use of biosurfactant has been limited due to the high production cost. Nevertheless, biosurfactants can be produced with high yield by some microorganisms, especially *Pseudomonas* sp. Some of these microorganisms can use the various renewal resources, such as agroindustrial wastes, animal fats, soapstocks, molasses, starch-rich wastes, potato substrates and olive oil mill effluent as the potential carbon sources. This leads to the greater possibility for economical biosurfactant production and reduced pollution caused by those wastes.

- Shah *et al.* 2005 [101] studied the effect of sophorolipid on spermicidal, anti-HIV, and cytotoxic activities. The sophorolipid diacetate ethyl ester derivative is the most potent spermicidal and virucidal agent of the series of SL studied. Its virucidal activity against HIV and sperm-immobilizing activity against human semen are similar to those of monoxynol-9. Interestingly, natural SLs and analogs thereof showed some degree of selectivity in their spermicidal, virucidal, and cytotoxic effects. For instance, the lactonic SL displayed high spermicidal, cytotoxic and proinflammatory activities but low virucidal activity. Conversely, open ring SLs were weak spermicides but potent virucides. The diacetyl ethyl ester SL displayed the highest spermicidal and anti-HIV activities. This study clearly demonstrated that certain sophorolipids and modified forms thereof have excellent spermicidal and anti-HIV activity.

- Mukherjee *et al.* 2006 [137] reviewed articles on towards commercial production of microbial surfactants. This article describes some practical approaches that have been adopted to make the biosurfactant production process economically attractive: these include the use of cheaper raw materials, optimized and efficient bioprocesses techniques and over producing mutant and recombinant strains for obtaining maximum productivity. They also reviewed use of cheaper substrates and effective down stream processing for efficient product recovery. The application of these strategies in biosurfactant production processes, particularly those using hyper – producing recombinant strains in the optimally controlled environment of a bioreactor, might lead towards the successful commercial production of these valuable and versatile biomolecules in near future.

- Langer *et al.* 2006[138] produced a mixture of oligosaccharide lipids from soil bacterium *Tsukamurella* Species DSM 44370, when cultivated on sunflower oil. In contrast cultivation with calendula oil as carbon source afforded a different product composition with overproduction of 2,3-di-o – acyl – β-D-glucopyranosyl – (1 -2) – β – D- galactopyranosyl – (1 -6) – 4- 6 di- o – acyl – α- D- glucopyranosyl – (1-1) – α – D – glucopyranose (GL 3) that amounted to

60% of the whole product. GL 3 and its parent tetrahexose backbone G3 were then modified enzymatically with the lipase Novozyme 435 from *Candida antarctica* by addition of one or two oleic acid molecules to GL 3 and four molecules to G3. The new glycolipids were shown to exhibit to reduce surface tension of water to 23 mN/m. In addition these products showed novel biological activities through the inhibition of the activation of Epstein-Barr virus early antigen.

- Jing *et al.* 2006 [99] isolated biosurfactant producing yeast strain, Y2A from oil-containing wastewater and identified as *Wickerhamiella domercqiae* by BIOLOG analysis and routine yeast identification method. The crude biosurfactants produced from Y2A were obtained by extract with ethyl acetate and proved to be a mixture of glycolipids by thin layer chromatography (TLC). The main product was separated and purified by HPLC and then characterized as sophorolipid by nuclear magnetic resonance (NMR) and mass spectroscopy (MS). This is the first report on sophorolipids produced from *W. domercqiae*. Subsequently, the cytotoxic effects of the sophorolipid on cancer cells of H7402, A549, HL60 and K562 were investigated by MTT assay. The results showed a dose-dependent inhibition ratio on cell viability according to the drug concentration ≤62.5_g/ml. These findings suggested that the sophorolipid produced by *W. domercqiae* have anticancer activity.

- Muller *et al.* 2006[100] studied sophorolipids decrease IgE production in U266 Cells and improves sepsis survival. They suggested that sophorolipids, a family of natural and easily chemo- enzymatically modified microbial glycolipids are promising modulators of immune response. They demonstrated that the sophorolipids possesses anti inflammatory effects, including decreasing sepsis related mortality at 36 h *in vivo* in a rat model.

- Nitschke and Costa 2007[35] reviewed the applications of biosurfactants in food industry. This review includes classification, properties of biosurfactants such as surface and interface activity, temperature, pH and ionic strength tolerance, biodegradability, low toxicity, emulsion forming and emulsion breaking,

antimicrobial activity and their potential food applications. The increasing environmental concern about chemical surfactants triggers attention to microbial derived surface active compounds essentially due to their low toxicity and biodegradable nature. At present, biosurfactants are predominantly used in remediation of pollutants; however, they show potential applications in many sectors of food industry. Associated with emulsion forming and stabilization, antiadhesive and antimicrobial activities are some properties of biosurfactants which could be explored in food processing and formulation. The use of agro industrial wastes as alternative substrates for their production is discussed.

- Felse *et al.* 2007[139] investigated the influence of fatty acid carbon chain length, unsaturation, source of low-cost industrial lipid feed stocks, and nickel content in lipid feed-stocks on sophorolipid production by *Candida bombicola.* Saturated and monounsaturated C18 fatty acid gave the highest levels of sophorolipid production while fatty acids with more than one site of unsaturation gave much lower yields.The possibility of exploiting low-cost industrial wastes or by-products for SL production by batch or fed-batch process was investigated Tallow fatty acid residue was the best lipid-feed stock for sophorolipid production (120 gL^{-1}, fed-batch cultures), while coconut fatty acid residue resulted in the lowest production (40 gL^{-1}, fed-batch cultures). *Candida bombicola* was tolerant to nickel contamination up to 112.5 mgL^{-1} during sophorolipid production. Sophorolipid product obtained from nickel contaminated lipid wastes had low nickel levels (<5 mgL^{-1}) that could allow its use in low-end consumer products and for household applications.

5. MATERIALS AND METHODS

5.1. MATERIALS

Peptone	Himedia labs ltd,Mumbai
Malt extract	Himedia labs ltd,Mumbai
Yeast extract powder type-I	Himedia labs ltd,Mumbai
Agar agar	Himedia labs ltd,Mumbai
Urea	Ranbaxy labs,sas nagar.
Glucose	S.d fine chemicals ltd,mumbai
Dextrose	Loba chemie
Maltose	S.d fine chemicals ltd, Mumbai
Lactose	S.d fine chemicals ltd, Mumbai
Sucrose	S.d fine chemicals ltd,Mumbai
Oleic acid	Indian research products,Chennai.
Ethyl acetate	S.d fine chemicals ltd,Mumbai
Petroleum ether	S.d fine chemicals ltd,Mumbai
n-hexane	S.d fine chemicals ltd,Mumbai
Aluminium pre-coated TLC plates	Merck India (pvt),Mumbai.
Sunflower oil	S&S pvt ltd
Soya bean oil	S&S pvt ltd
Coconut oil	S&S pvt ltd
Sodium chloride	S.d fine chemicals ltd,Mumbai
Potassium chloride	S.d fine chemicals ltd,Mumbai
Ammonium chloride	S.d fine chemicals ltd,Mumbai
Ammonium nitrate	S.d fine chemicals ltd,Mumbai
Sodium nitrate	S.d fine chemicals ltd,Mumbai
Potassium nitrate	Ranbaxy labs,sas nagar.
n-hexadecane	Himedia labs ltd,Mumbai

5.2. INSTRUMENTS AND EQUIPMENTS

Hotair oven	Technico
Incubator	Technico
Autoclave	Kailash
Horizontal laminar flow hood	Clean air Chennai.
Centrifuge	Remi Motors
Digital balance	Shimadzu
Rotary shaker	Remi motors
Heating mantle	Guna enterprises
Gel documentation	Alpha digidoc
Digital microscope	Motic

5.3. MICROORGANISMS

The yeasts employed in this work were *Candida tropicalis* NCIM 3120, *Candida tropicalis* NCIM 3121, *Candida tropicalis* NCIM 3122, *Candida tropicalis* NCIM 3123, *Candida rugosa* NCIM 3462, *Candida rugosa* NCIM 3592, *Candida lipolytica* NCIM 3472, *Candida lipolytica* NCIM 3229, *Candida lipolytica* NCIM 3450, *Candida bombicola* MTCC 1910.

5.4. PROFILE OF THE ORGANISM[140]

5.4.1. Taxonomic classification

Kingdom : Fungi

Phylum : Ascomycota

Subphylum : Ascomycotina

Class : Ascomyce

Order : Saccharomycetales

Family : Saccharomycetaceae

Genus : *Candida*

5.4.2. Description and natural habitates

Candida is yeast which is the most common cause of opportunistic mycoses worldwide. It is also a frequent colonizer of human skin and mucous membranes. *Candida* is a member of normal flora of skin, mouth, vagina and stool. Apart from being a pathogen and a colonizer, it is found in the environment,

particularly on leaves, flowers, water and soil. While most of the *Candida* species are mitosporic, some have known to be telemorphic and produce sexual spores.

5.4.3. Pathogenicity and clinical significance

Infections caused by *Candida* species are in general referred to as Candidiasis. The clinical sputum of patients with Candidiasis is extremely diverse. Almost any organ or system in the body can be affected. Candidiasis may be superficial and local or deep seated and disseminated. Disseminated infections arise from hematogenous, spread from the primarily infected locus. *Candida ablicans* is the most pathogenic and most commonly encountered species among all the species of this genus. Its ability to adhere to host tissues, to produce secretory aspartyl proteases and phospholipase enzymes, and its capacity to transform from yeast to hyphal phase are the major determinants of its pathogenicity. Several of the following host factors predispose to candidiasis:

Predisposing factor	**Examples**
Physiological	Pregnancy, age (elderly)
Trauma	Maceration, infection burn wound
Hematological	Neutropenia – cellular immunodeficiency
Endocrinological	Diabetes mellitus, hypoparathyrodism, Addison's disease
Iatrogenic	Chemotherapeutic agents, corticosteroids, Oral contraceptives, antibiotics, catheters
Others	Intravenous drug addition, malnutrition, malabsorption

5.4.4. Macroscopic and microscopic features of *Candida*

The colonies of *Candida* species are cream to yellow in colour and they grow rapidly and mature in 3 days. The texture of the colony may be pasty, smooth, or dry, wrinkled and dull, depending on the species. All the species produce blastoconidia singly or in small clusters. Blastoconidia may be round or elongate. Most species produce pseudohyphae which may be long, branched or curved. True hypae and chlamydosphores are produced by strains of some *Candida* species. Although they are the members of the same genus, the various

species do have some degree of unique behaviour with respect to their colony texture.

5.5. METHODS

5.5.1. Source and culture conditions

The different species of *Candida* were obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The culture was maintained on MGYP agar slant medium with monthly transfer.

Ingredients	Quantity
Glucose	1%
Malt extract	0.3%
Yeast extract	0.3%
Peptone	0.5%
Agar	2.0%
Distilled H_2O upto	100 ml
pH	6.4 to 6.8

Candida bombicola MTCC 1910 was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. This culture was maintained on a YEPD agar slant medium with monthly transfer.

Ingredients	Quantity
Yeast extract	0.3%
Peptone	1.0%
Dextrose	2.0%
Agar	1.5%
Distilled H_2O upto	100 ml
pH	6.4 to 6.8

5.5.2. Preparation of inoculum

Candida species

The organisms *Candida tropicalis* NCIM 3120, *Candida tropicalis* NCIM 3121, *Candida tropicalis* NCIM 3122,*Candida tropicalis* NCIM 3123,*Candida rugosa* NCIM 3462, *Candida rugosa* NCIM *3592, Candida lipolytica* NCIM 3472,*Candida lipolytica* NCIM 3229,*Candida lipolytica* NCIM 3450, were inoculated individually into the100ml broth containing malt extract 3 gL^{-1}, glucose 10 gL^{-1}, yeast extract 3 gL^{-1} and peptone 5 gL^{-1}, pH was adjusted to 5.0 to 5.6. The medium was sterilized by autoclaving before inoculation and then inoculated media was incubated for 48 h at 30°C.

***Candida bombicola* MTCC 1910**

The yeast, *Candida bombicola* MTCC1910 was inoculated into the 100ml broth containing yeast extract 3 gL^{-1}, peptone 10 gL^{-1} and dextrose 20 gL^{-1}. The medium was sterilized by autoclaving before inoculation and then incubated after inoculation for 48 h at 30°C. The above inoculum was used for further studies.

5.6. SCREENING FOR BIOSURFACTANT PRODUCING MICROORGANISM

Three methods to be used to screen for biosurfactant producing microorganism. They are evaluation of hemolysis, biosynthesis of biosurfactant from different medium and reduction of surface tension of culture broth.

5.6.1. Hemolysis

Hemolysis was used as an initial selection criterion for the primary isolation of surfactant - producing microorganism [141].

Method

Blood agar medium contained 40 gL^{-1} blood agar base, 50 mlL^{-1} of defibrinated sheep's blood with the pH adjusted to 5.6 ± 0.2 was used. The blood agar was prepared by adding medium components, except sheep blood, to distilled and deionized- water and bringing the volume to 950 ml. The product was mixed thoroughly, gently heated and brought to boiling. It was sterilized by autoclaving for 15 min at 15 psi pressure at 121^0C, and then cooled to 45^0 to 50^0C. Then 50 ml of sterile sheep blood was aseptically added and mixed thoroughly, poured into sterile petri dishes in 20ml volume. A loopful of the inoculum was taken and single streaking and multiple streaking were done and the plates were incubated for 24 h and the results were graded as α, β, and γ hemolysis[154] (Table 6). The results are presented in Fig.8 and 9.

Types of Hemolysis

a. Alpha (α) hemolysis: Alpha (α) hemolytic organisms produce a zone of partial clearing (greening) around the colonies.

b. Beta (β) hemolysis: Beta (β) hemolytic organisms produce complete zone of clearing around the colony.

c. Gamma (γ) hemolysis: Some micro organisms grow on blood agar with no hemolysis.

Table 6

Hemolysis of different species of *Candida*

S. No.	Name of Microorganism	Type of Hemolysis
1	*Candida tropicalis* NCIM 3120	α
2	*Candida tropicalis* NCIM 3121	γ
3	*Candida tropicalis* NCIM 3122	α
4	*Candida tropicalis* NCIM 3123	γ
5	*Candida rugosa* NCIM 3462	γ
6	*Candida rugosa* NCIM 3592	γ
7	*Candida rugosa* NCIM 3472	γ
8	*Candida lipolytica* NCIM 3472	γ
9	*Candida lipolytica* NCIM 3229,	γ
10	*Candida lipolytica* NCIM 3450	γ
11	*Candida bombicola* MTCC1910	β

✪ α - Partial hemolysis, ✪ β- Complete hemolysis ✪ γ - No hemolysis

5.6.2. Determination of surface tension

Reduction of surface tension in culture broth during fermentation is one of the important criteria for identification of biosurfactant producing species.

Sophorolipid production

Medium consisted of glucose 100 gL^{-1}, yeast extract 10 gL^{-1}, urea 1 gL^{-1}, oleic acid 40 gL^{-1}, and was sterilized by autoclaving (121°C for 20 minutes). 50 ml filter - sterilized lipid (oleic acid) was added to the flasks. The sterile fermentation medium (300 ml) was inoculated with 30% v/v inoculum. Agitation set at 200 rpm. Temperature was maintained at 30°C and pH was not controlled during fermentation. During the fermentation, 40 ml of culture broth was aseptically removed from the batch culture for every 24 h till 168 h. The collected culture broth was centrifuged at 5000 rpm for 30 min. The cell free supernatant containing sophorolipids was used for determination of surface tension by stalagnometer. The results are presented in Table 7.

Table 7

Determination of surface tension of culture broth during sophorolipid production using different species of *Candida*.

Organism	Surface tension of culture broth (dynes cm^{-1})						
	24h	**48h**	**72h**	**96 h**	**120h**	**144h**	**168h**
Candida tropicalis NCIM 3120	52.73	52.72	52.78	52.78	52.75	52.74	52.77
Candida tropicalis NCIM *3121*	52.76	52.76	52.74	52.72	52.75	52.77	52.70
Candida tropicalis NCIM 3122	52.71	52.77	52.72	52.75	52.78	52.76	52.74
Candida tropicalis NCIM 3123	52.65	52.67	52.64	52.60	52.65	52.67	52.68
Candida rugosa NCIM 3462	52.72	52.78	52.70	52.77	52.75	52.74	52.76
Candida rugosa NCIM 3592	52.75	52.77	52.78	52.74	52.74	52.72	52.76
Candida lipolytica NCIM 3472	52.04	52.06	52.08	52.02	52.07	52.08	52.03
Candida lipolytica NCIM 3229	52.57	52.58	52.58	52.56	52.53	52.58	52.54
Candida lipolytica NCIM 3450	53.51	53.34	53.42	53.45	53.44	53.43	53.48
Candida bombicola MTCC 1910	52.16	49.74	48.38	46.50	42.57	40.15	40.14

5.6.3. Sophorolipid biosynthesis

To screen the sophorolipid producing organisms by using following different screening media which were prepared as per the details collected from the literature.

Screening medium

Medium 1 [139]

- Glucose - 100 gL^{-1}
- Yeast extract - 10 gL^{-1}
- Urea - 1 gL^{-1}
- Oleic acid - 40 gL^{-1}

Medium 2

- Glucose - 100 gL^{-1}
- Yeast extract - 10 gL^{-1}
- Urea - 1 gL^{-1}
- Soyabean oil- 40 gL^{-1}

Medium 3

- Glucose - 100 gL^{-1}
- Yeast extract - 10 gL^{-1}
- Urea - 1 gL^{-1}

Medium 4

- Glucose - 100 gL^{-1}
- Hexadecane 100 gL^{-1}

Medium 5

- Glucose - 100 gL^{-1}
- Urea - 1 gL^{-1}

After sterilization of the above media, 10% v/v inoculum was added and fermentations were carried out in 500 ml Erlenmeyer flask containing 100 ml screening medium for 168 h, at 30 ± 1°C in a rotary shaker at 120 rpm. At the beginning of the fermentation the pH of this medium was adjusted to 5.6 and it was not being controlled during the process. All the other media compositions and culture conditions remained the same for other species.

EXTRACTION OF SOPHOROLIPIDS [76]

To isolate sophorolipids the entire culture (cells and broth) was lyophilized for 8 h. The dried residues were divided into six portions. Each portion was extracted with ethyl acetate (500 ml) by shaking at 30^0C for 5 days. The extraction mixture was filtered through Whatman No. 2 filter paper, and the residues were rinsed twice with ethyl acetate (500 ml each time). The combined filtrate was concentrated by evaporation and added to 1, 1 hexane/petroleum ether (90/10 v/v) to precipitate out the pure sophorolipids. After vacuum drying in a desiccator, the sophorolipids were weighed. The yields are presented in table 8-12.

Table 8

Summary of biosurfactant production studies with *Candida* species by using medium 1

Organism	Initial glucose gL^{-1}	Yield of SL gL^{-1}	Surface tension of culture broth (dynes cm^{-1})
Candida tropicalis NCIM *3120*	100	NY	56.44
Candida tropicalis NCIM *3121*	100	NY	52.20
Candida tropicalis NCIM 3122	100	NY	56.47
Candida tropicalis NCIM 3123	100	NY	56.42
Candida rugosa NCIM 3462	100	NY	56.48
Candida rugosa NCIM 3592	100	NY	56.49
Candida lipolytica NCIM 3472	100	NY	56.45
Candida lipolytica NCIM 3229	100	NY	56.48
Candida lipolytica NCIM 3450	100	NY	56.43
Candida bombicola MTCC 1910	100	56.3	32.36

Table 9

Summary of biosurfactant production studies with *Candida* species by using medium 2

Organism	Initial glucose gL^{-1}	Yield of SL gL^{-1}	Surface tension of culture broth (dynes cm^{-1})
Candida tropicalis NCIM *3120*	100	NY	56.44
Candida tropicalis NCIM *3121*	100	NY	52.20
Candida tropicalis NCIM 3122	100	NY	56.47
Candida tropicalis NCIM 3123	100	NY	56.42
Candida rugosa NCIM 3462	100	NY	56.48
Candida rugosa NCIM 3592	100	NY	56.49
Candida lipolytica NCIM 3472	100	NY	56.45
Candida lipolytica NCIM 3229	100	NY	56.48
Candida lipolytica NCIM 3450	100	NY	56.43
Candida bombicola MTCC 1910	100	54.2	35.62

Table 10

Summary of biosurfactant production studies with *Candida* species by using medium 3

Organism	Initial glucose gL^{-1}	Yield of SL gL^{-1}	Surface tension of culture broth (dynes cm^{-1})
Candida tropicalis NCIM *3120*	100	NY	56.44
Candida tropicalis NCIM *3121*	100	NY	52.20
Candida tropicalis NCIM 3122	100	NY	56.47
Candida tropicalis NCIM 3123	100	NY	56.42
Candida rugosa NCIM 3462	100	NY	56.48
Candida rugosa NCIM 3592	100	NY	56.49
Candida lipolytica NCIM 3472	100	NY	56.45
Candida lipolytica NCIM 3229	100	NY	56.48
Candida lipolytica NCIM 3450	100	NY	56.43
Candida bombicola MTCC 1910	100	34.6	36.52

Table 11

Summary of biosurfactant production studies with *Candida* species by using medium 4

Organism	Initial glucose gL^{-1}	Yield of SL gL^{-1}	Surface tension of culture broth (dynes cm^{-1})
Candida tropicalis NCIM *3120*	100	NY	56.44
Candida tropicalis NCIM 3121	100	NY	52.20
Candida tropicalis NCIM 3122	100	NY	56.47
Candida tropicalis NCIM 3123	100	NY	56.42
Candida rugosa NCIM 3462	100	NY	56.48
Candida rugosa NCIM 3592	100	NY	56.49
Candida lipolytica NCIM 3472	100	NY	56.45
Candida lipolytica NCIM 3229	100	NY	56.48
Candida lipolytica NCIM 3450	100	NY	56.43
Candida bombicola MTCC 1910	100	41.3	36.69

Table 12

Summary of biosurfactant production studies with *Candida* species by using medium 5

Organism	Initial glucose gL^{-1}	Yield of SL gL^{-1}	Surface tension of culture broth (dynes cm^{-1})
Candida tropicalis NCIM 3120	100	NY	56.44
Candida tropicalis NCIM 3121	100	NY	52.20
Candida tropicalis NCIM 3122	100	NY	56.47
Candida tropicalis NCIM 3123	100	NY	56.42
Candida rugosa NCIM 3462	100	NY	56.48
Candida rugosa NCIM 3592	100	NY	56.49
Candida lipolytica NCIM 3472	100	NY	56.45
Candida lipolytica NCIM 3229	100	NY	56.48
Candida lipolytica NCIM 3450	100	NY	56.43
Candida bombicola MTCC 1910	100	36.18	35.96

5.7. CULTURAL AND BIOCHEMICAL CHARACTERIZATION OF *Candida bombicola* MTCC 1910

Candida bombicola MTCC 1910 was identified as biosurfactant producing species by the studies of hemolysis, determination of surface tension of culture broth and biosynthesis of sophorolipids using five different screening media and selected *Candida bombicola* MTCC 1910 were subjected to biochemical characterization by conventional method.

5.7.1. MACROSCOPIC MORPHOLOGY[142]

The yeast *Candida bombicola* MTCC 1910 was inoculated into Sabouraud dextrose broth and gently swabbed into Sabouraud dextrose agar and incubated for 48h and observed for their growth pattern.

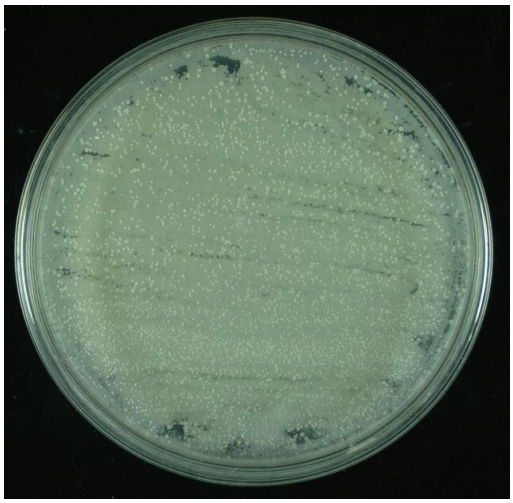


Fig. 10

Growth pattern of *Candida bombicola* on Sabouraud dextrose agar

5.7.2. MICROSCOPIC MORPHOLOGY OF YEASTS [142]

Two wet mounts of the yeast culture were prepared from 5 days culture of Sabouraud agar in the following

a. A loopful of yeast culture was suspended in to a few drops of the water – iodine solution on a microscope slide and covered with a coverslip.

b. A loopful of yeast culture was mixed in a few drops of lactophenol cotton - blue solution on a microscope slide and covered with a coverslip.

Both the yeast slide preparations were examined under microscope. The photographs are shown in Fig. 10a-10d.

Fig.10 a: Lactophenol cotton blue stain: *Candida bombicola* MTCC 1910 (10x)

Fig.10 b: Lactophenol cotton blue stain: *Candida bombicola* MTCC 1910 (10x)

Fig.10 c: Lactophenol cotton blue stain: *Candida bombicola* MTCC 1910 (40x)

Fig.10 d: Water iodine stain: *Candida bombicola* MTCC 1910 (10x)

5.7.3. UREASE TEST

Urease test was done to determine the ability of microorganisms to degrade urea by means of the enzyme urease. Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product ammonia.

Procedure

1. Using sterile technique, inoculate the organism into the urea broth by means of loop inoculation.
2. Incubate the culture for 24 to 48hours at 37^0C.
3. Results are recorded in table 13 and photographs are shown in fig.11.

5.7.4. CARBOHYDRATE FERMENTATION

Carbohydrate fermentation was performed to determine the ability of the microorganisms to degrade and ferment carbohydrates with the production of an acid or acid and gas.

Procedure

1. Using sterile technique, inoculate the organism into the phenol red broth containing glucose, by means of loop inoculation. Taking care during this step not to shake the fermentation, shaking the tube may accidentally force a bubble of air into the Durham's tube, displacing the medium and possible rendering a false positive result. The glucose can be replaced with other carbohydrates like dextrose, lactose, maltose, sucrose etc and the same procedure was followed.
2. Incubate all of the tubes for 24 to 48 hours at 37^0C.
3. Results are shown in table 13 and photographs are presented in fig.11a-11e.

5.7.5. GERM TUBE TEST[143]

The identification of *Candida* species is usually based on its ability to produce short, slender tube like structures called germ tubes when incubated at 37°C for 2 to 4 h in pooled human sera. This is most commonly used method for performing germ tube test.

The yeast was lightly inoculated into 0.5 mL of pooled normal human serum and incubated for 2 h at 37^0 C. At the two hour limit, one or two drops of suspension was placed on a clear glass slide and mounted with a cover slip. The slide preparation was examined for the presence of germ tube. A positive control (*Candida albicans)* and a negative control were used to help interact the result. Results are presented in table 13.

5.7.6. CARBOHYDRATE ASSIMILATION TEST

1. 2 mm loopful of pure culture was suspended in 9.0 ml of sterile water.
2. The assimilation slant was inoculated with 0.1ml of suspension.
3. The slants were incubated at room temperature and were observed at 7 and 14 days for abundant growth and acid production (Yellow), results are shown in table 13.

Table 13

Assimilation, Fermentation and Urease test for *Candida bombicola* MTCC 1910

Species	Urease test	Assimilation of					Fermentation of					Germ tube test
		D-Glucose	Dextrose	Sucrose	Lactose	Maltose	D-Glucose	Dextrose	Sucrose	Lactose	Maltose	
***Candida bombicola* MTCC 1910**	+	+	-	+	+	-	+	+	+	+	+	+

Fig.11: UREASE ACTIVITY:
1. Control
2. *Candida bombicola* MTC 1910: *Positive*

Fig. 11 a:CARBOHYDRATE FERMENTATION:(Glucose)
1. Control
2. *Candida bombicola* MTC 1910: Acid and gas

Fig.11 b: CARBOHYDRATE FERMENTATION :(Dextrose)
1. Control
2. *Candida bombicola* MTC 1910: Acid

Fig.11 c: CARBOHYDRATE FERMENTATION: (Sucrose)
1. Control
2. *Candida bombicola* MTC 1910*: Acid* and gas

Fig.11 d: CARBOHYDRATE FERMENTATION: (Maltose)
1. Control
2. *Candida bombicola* MTC 1910*: Acid*

Fig.11 e: CARBOHYDRATE FERMENTATION :(Lactose)
1. Control
2. *Candida bombicola* MTC 1910: Acid.

5.8. OPTIMIZATION OF MEDIUM COMPOSITION

CONDITIONS OF MEDIUM COMPOSITION

Candida bombicola MTCC 1910 was used in the optimization of medium composition and cultural conditions for sophorolipid production. Medium composition was determined by shake flask method using Erlenmeyer flask in a rotary shaker.

5.8.1. CARBON SOURCE

The effect of different carbon sources and their concentration on the production of sophorolipids by *Candida bombicola* MTCC 1910 was determined by fermentation medium containing only carbon source in batch fermentation using shake flask method.

Sophorolipid production

The fermentation medium was composed of 100g/L glucose. Medium was sterilized at the temperature of 121^0C for 20 minutes and incubated with 10% inoculum. Batch culture was carried out in 500 ml Erlenmeyer flask containing 100ml production medium for 168 hours at 30^0C ± 1^0C in a rotary shaker at 120 rpm. During fermentation pH was not controlled. The sophorolipids were extracted with ethyl acetate. The production media and cultural conditions were

same for other carbon sources. D-glucose was replaced with other carbon sources such as sucrose, dextrose, lactose, maltose, n-hexadecane and their concentration also studied. The results were given in table 14.

Table 14

Influence of various carbon sources on sophorolipid production by *Candida bombicola* MTCC 1910 in batch culture.

Species	**Sophorolipid yield in g/L**					
	Glucose	**Dextrose**	**Sucrose**	**Lactose**	**n-hexadecane**	**Maltose**
Candida bombicola MTCC 1910	NY	NY	NY	NY	NY	NY

NY-No Yield

Fig. 12: Production of sophorolipid from *Candida bombicola* MTCC 1910 by shake flask method

5.8.2. NITROGEN SOURCE

The influence of nitrogen sources and their concentration on sophorolipid production by *Candida bombicola* MTCC 1910 was studied by batch fermentation. Various nitrogen sources such as potassium nitrate, sodium nitrate, ammonium nitrate, urea, yeast extract, peptone were studied.

Sophorolipid production

The production medium composed of 100g/L glucose and 10g/L of potassium nitrate. Medium sterilization and cultural conditions were as same as that of carbon source optimization. Potassium nitrate was replaced with other nitrogen sources in fermentation media. The effect of nitrogen sources and the effect of combination of organic and inorganic nitrogen sources were also studied for *Candida bombicola* MTCC 1910. The sophorolipids were isolated by extracting with ethyl acetate. The yields are given in table 15.

Table 15

Influence of various nitrogen sources on sophorolipids production by *Candida bombicola* MTCC 1910 in batch culture.

Species	Sophorolipid yield in g/L						
	(10 g/L)Potassium nitrate	Sodium nitrate (10 g/L)	(10 g/L)Ammonium nitra	Urea (10 g/L)	Yeast extract (10 g/L)	Peptone (10 g/L)	Urea + yeast extract (10 g/L)
Candida bombicola MTCC 1910	26.56	20.75	9.75	41.25	42.04	17.96	58.26

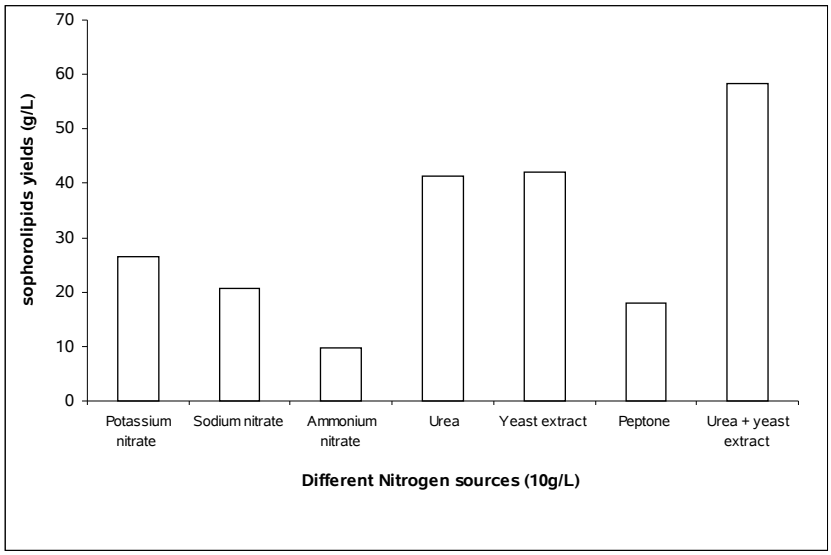


Fig.13

Relationship between different nitrogen sources and sophorolipid yields for *Candida bombicola* MTCC 1910

5.8.3. LIPID SOURCE

The influences of lipid source and on the production of sophorolipids were studied by *Candida bombicola* MTCC 1910.Various lipid sources such as oleic acid, soyabean oil, sunflower oil, coconut oil were studied.

Sophorolipid production

The production medium compound of 100 g/L of glucose and 10 g/L of urea, 10 g/L of Yeast extract and 40 ml/L of oleic acid. Medium sterilization and other cultural conditions are same as mentioned above. Oleic acid was replaced with other lipid sources in fermentation media. The sophorolipids were isolated by extracting with ethyl acetate. The yields are given in the table16.

Table 16

Influence of various lipid sources on sophorolipid production by *Candida bombicola* MTCC 1910 in batch culture.

Species	Sophorolipid yield in g/L			
	Oleic acid (40ml/L)	**Soya bean oil (40ml/L)**	**Sunflower oil (40ml/L)**	**Coconut oil (40ml/L)**
Candida bombicola MTCC 1910	63.43	35.86	19.26	15.33

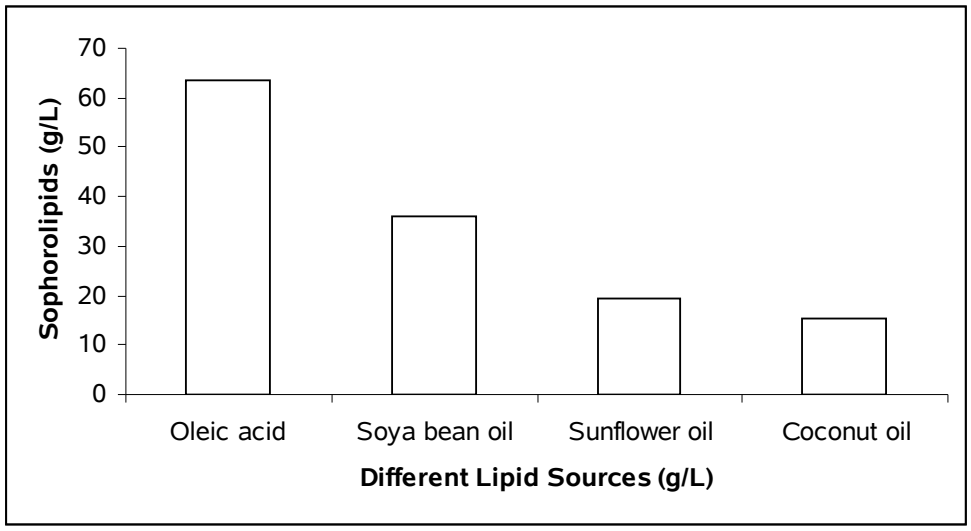


Fig. 14

Relationship between different lipid sources and sophorolipid yields for *Candida bombicola* MTCC 1910

5.8.4. EFFECT OF VITAMINS AND N-HEXADECANE

The influences of vitamins, n-hexadecane on the production of sophorolipids were studied by *Candida bombicola* MTCC 1910 by batch fermentation.

Sophorolipid production

The production medium composed of 100 g/L of glucose, 10 g/L urea, 10 g/L yeast extract and 10 ml/L n-hexadecane. Medium sterilization and other cultural conditions were same as mentioned above. n-hexadecane was replaced with other substrates in the fermentation media**.**

Table 17

Effect of vitamins, n-hexadecane on sophorolipid production by *Candida bombicola* MTCC 1910 in batch culture.

Species	Sophorolipid yield in g/L	
	n-hexadecane	Vitamins
Candida bombicola MTCC 1910	61.56	60.86

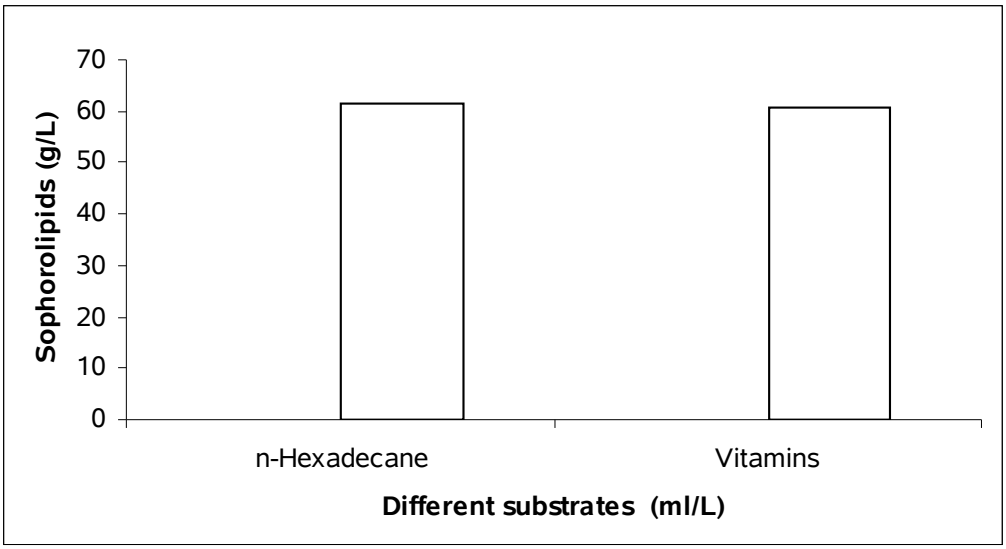


Fig. 15

Relationship between different substrates and sophorolipid yields for *Candida bombicola* MTCC 1910

5.8.5. EFFECT OF MINERAL SALTS

The effects of mineral salts on the production of sophorolipid were studied by *Candida bombicola* MTCC 1910 on various mineral salts such as sodium chloride, Ammonium chloride and Potassium chloride.

Sophorolipid production

The production medium composed of 100 g/L of glucose, 10 g/L of urea, 10 g/L of yeast extract, 40 ml/L of oleic acid, 10 ml/L n-hexadecane and 2 g/L of NaCl. Medium sterilization and other cultural conditions were same as mentioned above. NaCl was replaced with other mineral salts in the fermentation media. The sophorolipid were isolated by extracting with ethyl acetate. The yields are given in the table 18.

Table 18

Influence of various mineral salts on sophorolipid production by *Candida bombicola* MTCC 1910 in batch culture.

Species	Sophorolipid yield in g/L		
	Sodium chloride	Ammonium chloride	Potassium chloride
Candida bombicola MTCC 1910	62.03	60.95	59.65

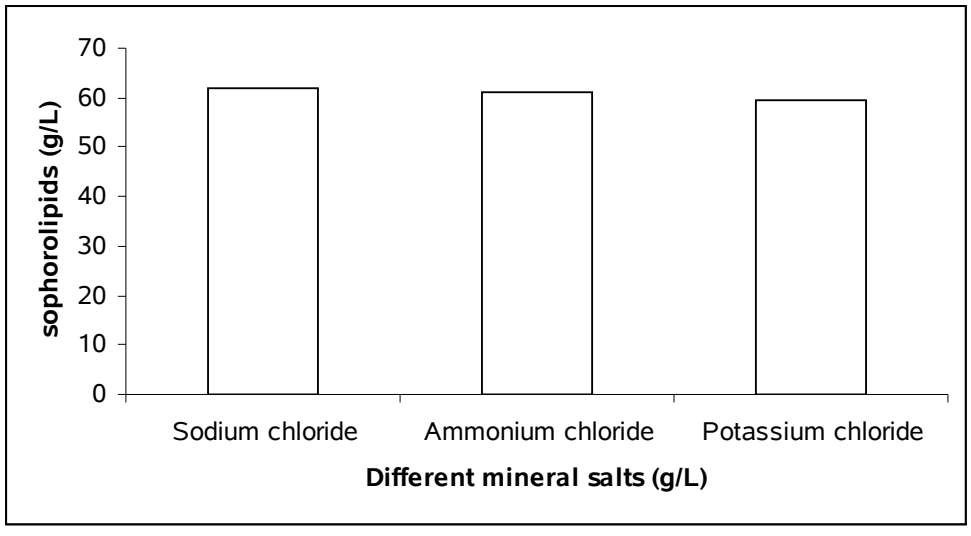


Fig. 16

Relationship between different mineral salts and sophorolipid yields for *Candida bombicola* MTCC 1910

Table 19

Optimized medium composition for *Candida bombicola* MTCC 1910

Ingredients	Quantity (g/L)
Glucose	100
Yeast extract	10
Urea	1
Oleic acid	40

5.9. OPTIMISATION OF CULTURAL CONDITIONS

5.9.1. EFFECT OF PH

The optimum pH for the fermentation process of *Candida bombicola* MTCC 1910 for the production of sophorolipid was determined by carrying out the various pH ranges from 2 to 6.

Sophorolipid production

The optimized fermentation medium (Table 19) and same cultural conditions are mentioned above was carried out in a 500 ml flasks in a digital orbital shaker at various pH such as 3, 3.5, 4, 4.5 and 5. The sophorolipids was isolated by extracting with ethyl acetate. The yield of sophorolipid of *Candida bombicola* MTCC 1910 are given in table 20.

Table 20

Effect of different pH on sophorolipid production by *Candida bombicola* MTCC 1910 in batch culture

Species	Sophorolipid yield in g/L				
	pH 3	pH 3.5	pH 4	pH 4.5	pH 5
Candida bombicola MTCC 1910	70.28	71.56	70.56	60.16	53.85

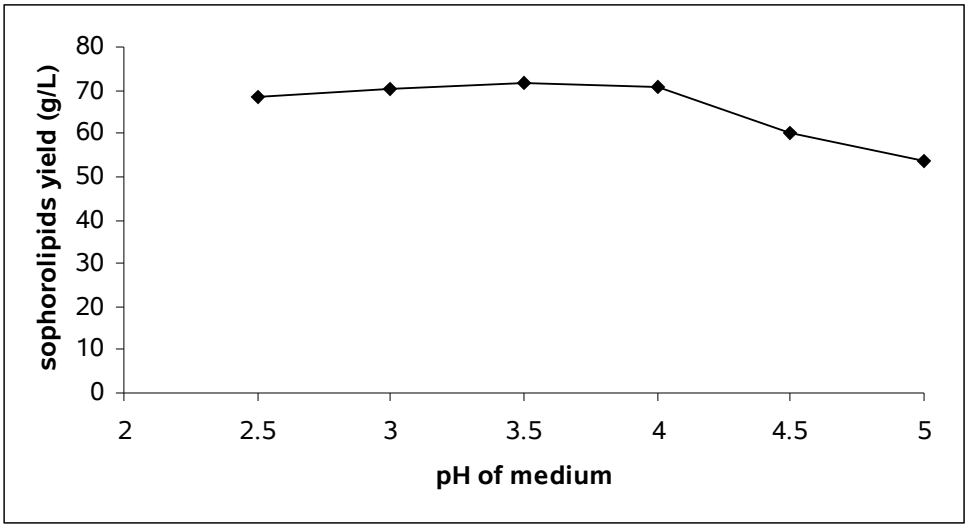


Fig. 17

Relationship between pH of medium and sophorolipids yields for *Candida bombicola* MTCC 1910

5.9.2. INCUBATION PERIOD

The optimum incubation period for the fermentation process of *Candida bombicola* MTCC 1910 for the production of sophorolipid was determined by carrying out the fermentation at various incubation periods.

Sophorolipid production

The optimized fermentation medium (Table19)and other cultural conditions are mentioned above was carried out in a 500 ml flasks in a digital orbital shaker at various incubation periods such as 96h, 168h, 240h and 312h. The sophorolipid was isolated by extracting with ethyl acetate. The yield of sophorolipid of *Candida bombicola* MTCC 1910 are given in table 21.

Table 21

Influence of various incubation periods on sophorolipid production by *Candida bombicola* MTCC 1910 by batch culture.

Species	Sophorolipid yield in g/L			
	Incubation Period 96 h	Incubation Period 168 h	Incubation Period 240 h	Incubation Period 312 h
Candida bombicola MTCC 1910	32.9	86.5	81.8	72.8

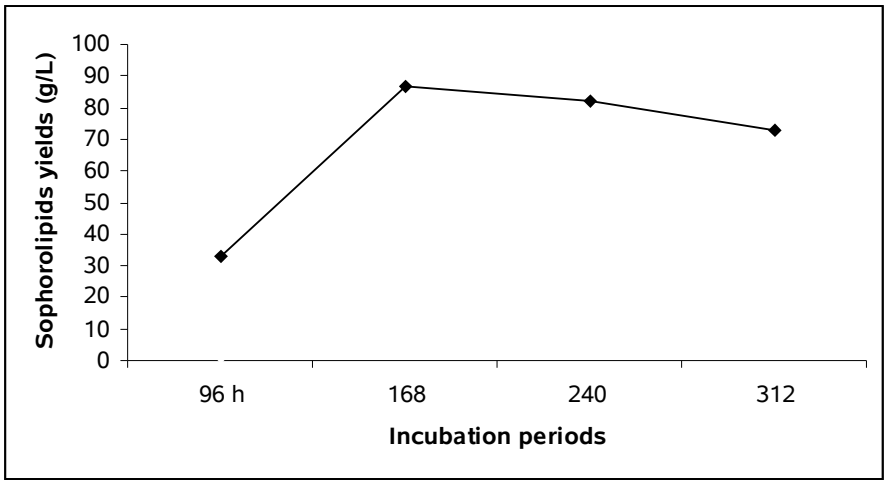


Fig. 18

Relationship between different incubation periods and sophorolipid yields for *Candida bombicola* MTCC 1910

5.9.3. SPEED OF AGITATION

The optimum speed of agitation for the fermentation process of *Candida bombicola* MTCC 1910 for the production of sophorolipid was determined by carrying out the fermentation at various speeds of agitation ranging from 120 to 220 rpm.

Sophorolipid production

The optimized fermentation medium (Table19) and other cultural conditions are mentioned above was carried out in a in a 500 ml flasks in a digital orbital shaker at various speeds of agitation such as 120, 150, 180, 200 and 220. The sophorolipid was isolated by extracting with ethyl acetate. The yields of sophorolipid of *Candida bombicola* MTCC 1910 are given in table 22.

Table 22

Influence of various speeds of agitation on sophorolipid production by *Candida bombicola* MTCC 1910 by batch culture.

Species	Sophorolipid yield in g/L				
	Speed of agitation 120 rpm	**Speed of agitation 150 rpm**	**Speed of agitation 180 rpm**	**Speed of agitation 200 rpm**	**Speed of agitation 220 rpm**
Candida bombicola MTCC 1910	65.4	72.5	79.0	85.2	78.6

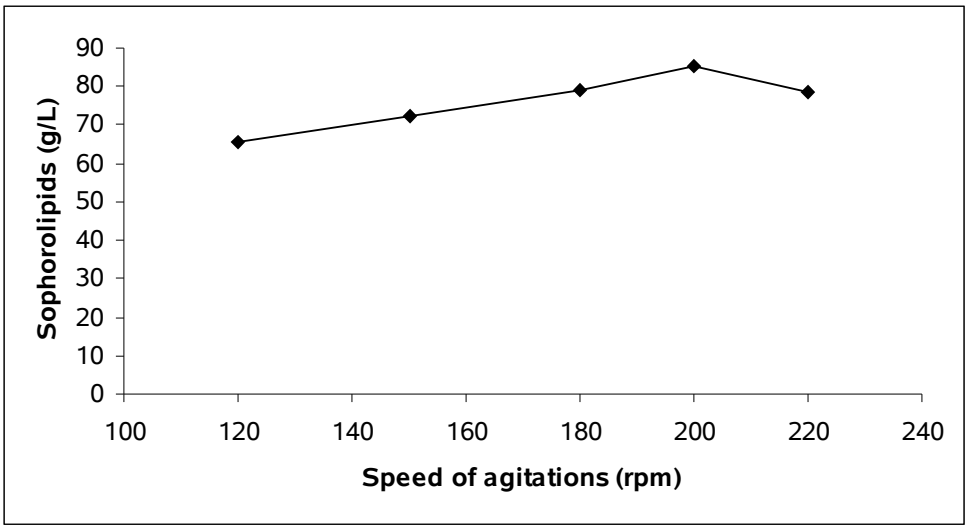


Fig. 19

Relationship between different speed of agitations and sophorolipid yields for *Candida bombicola* MTCC 1910

Table 23

Optimized cultural conditions for *Candida bombicola* MTCC 1910

Temperature	30^0C
pH	3.5
Incubation period	7 days
Rpm	200

5.10. FERMENTATIVE PRODUCTION OF SOPHOROLIPID BY OPTIMISED MEDIUM AND OPTIMISED CULTURAL CONDITIONS

5.10.1. BATCH CULTURE

Before inoculation the production medium was supplemented with 100 g of glucose, 10 g of yeast extract and 1 g of urea. The medium was sterilized in an autoclave before inoculation; 40ml/L of oleic acid (filter sterilized) was added to the medium after sterilization. 100 ml of inoculum was added to the 1000 ml of production medium and the fermentation was carried out 30^0C at 200 rpm at a constant pH 3.5 for 7 days. After 7 days the fermentation medium was removed and extraction was carried out.

5.10.2. FED BATCH CULTURE – I

The above-mentioned procedure as in batch culture was performed and after 3 days, 40g/L of glucose was sequentially added for every 24 h till the 144 h. The extraction was carried out after 7 days of fermentation.

5.10.3. FED BATCH CULTURE – II

The above-mentioned procedure as in batch culture was performed and after 3 days, 40 g/L of glucose and 20 g/L of oleic acid was sequentially added for every 24 hrs till 144 h. The extraction was carried out after 7 days of fermentation.

Extraction of sophorolipids [76]

To isolate sophorolipids the entire culture (cells and broth) was lyophilized for 8 hours. The dried residues were divided into six portions. Each portion was extracted with ethyl acetate (500 ml) by shaking at 30^0C for 5 days. The extraction mixture was filtered through Whatman No. 2 filter paper, and the residues were rinsed twice with ethyl acetate (500 ml each time). The combined filtrate was

concentrated by evaporation and added to 1,1 hexane/petroleum ether (90/10 v/v) to precipitate out the pure sophorolipids. After vacuum drying in a desiccator, the sophorolipids were weighed.The yields are presented in table 24.

Table 24

Sophorolipid yield for *Candida bombicola* MTCC 1910 by different fermentation methods

Process Strategy	Yield in g/g of glucose	Initial glucose g/L	Yield of Sophorolipid g/L
Batch culture	0.865	100	86.49
Fed Batch – I	1.003	100	90.85
Fed Batch – II	1.095	100	98.50

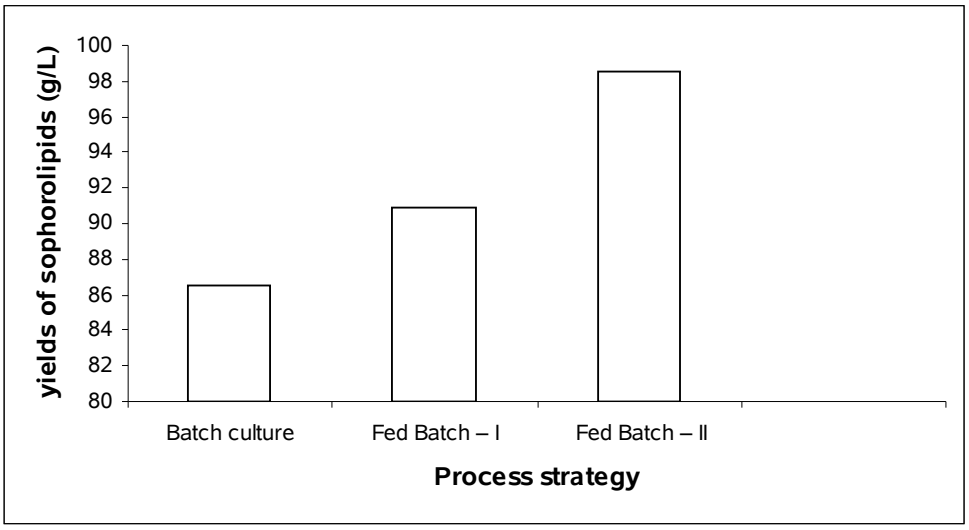


Fig. 20

Relationship between different process strategy and sophorolipid yields for *Candida bombicola* MTCC 1910

Fig. 21: Sophorolipid produced by *Candida bombicola* MTCC 1910 using batch culture

Fig.22: Sophorolipid produced by *Candida bombicola* MTCC 1910 using fed batch culture I

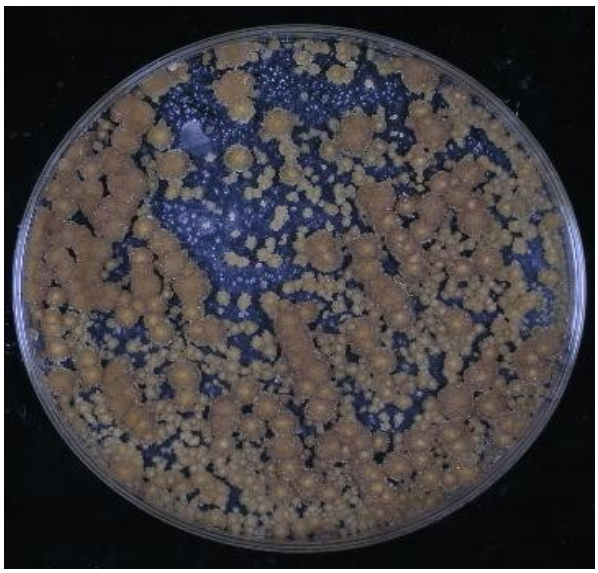


Fig.23: Sophorolipid produced by *Candida bombicola* MTCC 1910 using fed batch culture II

5.11. CHARACTERISATION OF SOPHOROLIPIDS [144]

The characterizations of sophorolipids were done by determining the surface tension, and critical micelle concentration (CMC).

5.11.1. DETERMINATION OF SURFACE TENSION (Drop count method)

The sophorolipids obtained from *Candida bombicola* MTCC 1910 were utilized for determining the surface tension by drop count method using stalagnometer. 40 ml of the solution containing 2% of microbial surfactant was used for determining the surface tension.

The surface tension was calculated using the formula.

$$r_2 = \frac{n_2 P_1}{n_1 P_2} x r_1$$

Where,

r_1 = Surface tension of water at 30^0C, 71.8 dynes/cm

r_2 = Surface tension of test liquid (medium containing the microbial surfactant)

n_1 = No. of drops of test liquid (medium containing the microbial surfactant)

n_2 = No. of drops of water

p_1 = Density of test liquid (medium containing the microbial surfactant)

P_2 = Density of water at 30^0C, 0.9956 g/cc

The density of test liquid was determined by the formula

$$P_1 = \frac{W_3 - W_1}{W_2 - W_1} x P_2$$

Where

W_1 = Weight of pyknometer in grams

W_2 = Weight of pyknometer + water in grams

W_3 = Weight of pyknometer + test liquid in grams

P_1 = Density of test liquid

P_2 = Density of water at 30^0C, 0.9956 g/cc

The results were tabulated in table 25.

Table 25

Determination of Surface tension for sophorolipids obtained from *Candida bombicola* MTCC 1910

S.No	Sample	Average no. of drops	Density (gms/cc)	Surface tension(dynes/cm)
1	Sophorolipid*	70	1.008	30.11
2	Span 80	78	1.016	27.02
3	Tween 40	75	1.005	28.10

*** Sophorolipid obtained from *Candida bombicola* MTCC 1910**

5.11.2. DETERMINATION OF CRITICAL MICELLE CONCENTRATION (CMC)

The concentration of the microbial surfactant at which it begins to form micelles was known as critical micelle concentration. Initially 2% stock solution was prepared by dissolving 2 ml of the partially purified microbial surfactant in 100ml of distilled water. Then 0%, 0.01%, 0.03%, 0.04%, 0.05%, 0.06%, 0.1%, 0.2% were obtained by taking 0,0.5,1,1.5,2,2.5,3,4,5,10 and 15 ml of stock solution and making it upto 100 ml in standard flask. The surface tensions of the various dilutions were determined. The CMC can be estimated by interpolating a plot of surface tension versus concentrations of sophorolipid. The results were shown in table 26.

5.11.3 Scanning Electron Micrograph of crude sophorolipids

The surface morphology and internal textures of crude sophorolipids obtained from *Candida bombicola* MTCC 1910 were observed under a scanning electron microscope. The photographs are given in Fig. 25a-25d .

Table 26

Determination of critical micelle concentration for sophorolipids obtained from *Candida bombicola* MTCC 1910

S.No	Concentrations of sophorolipids solutions (%)	Surface tension Dynes/cm	Critical micelle concentration mg/100 ml
1	0	72.09	30
2	0.01	51.31	
3	0.02	42.36	
4	0.03	32.69	
5	0.04	32.15	
6.	0.05	32.19	
7	0.06	32.06	

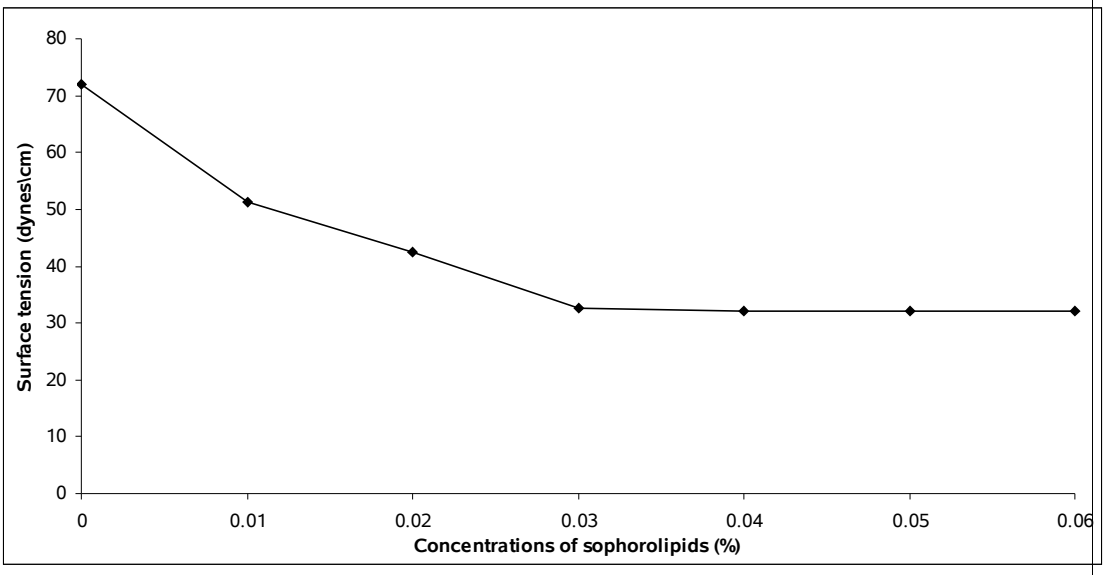


Fig. 24

CMC of Sophorolipid obtained from *Candida bombicola* MTCC 1910

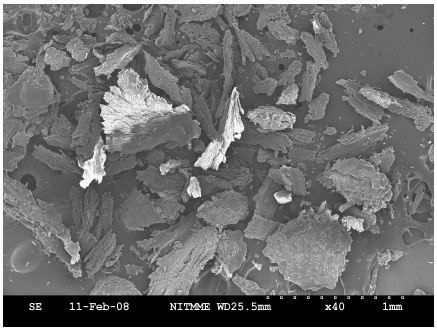


Fig. 25 a. SEM of sophorolipid obtained from *Candida bombicola* MTCC 1910 in X 40 magnification

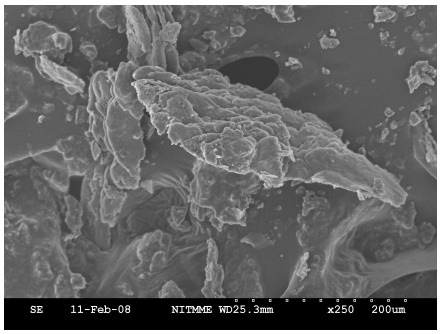


Fig. 25 b. SEM of sophorolipid obtained from *Candida bombicola* MTCC 1910 in X 250 magnification

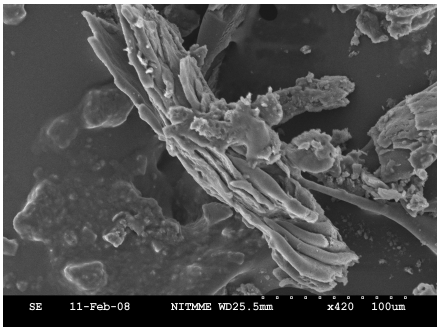


Fig. 25 c. SEM of sophorolipid obtained from *Candida bombicola* MTCC 1910 in X 420 magnification

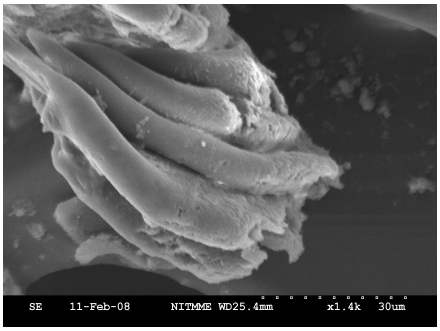


Fig. 25 d. SEM of sophorolipid obtained from *Candida bombicola* MTCC 1910 in X 1.4k magnification

5.12. SPECTRAL ANALYSIS OF SOPHOROLIPIDS:

Analysis of sophorolipids was performed by using the following methods:

- FTIR
- LC-APCI-MS

5.12.1. FTIR

A spectrum was recorded on JASCO FTIR-410 at pharmaceutical analysis laboratory, College of pharmacy, SRIPMS, Coimbatore-44.

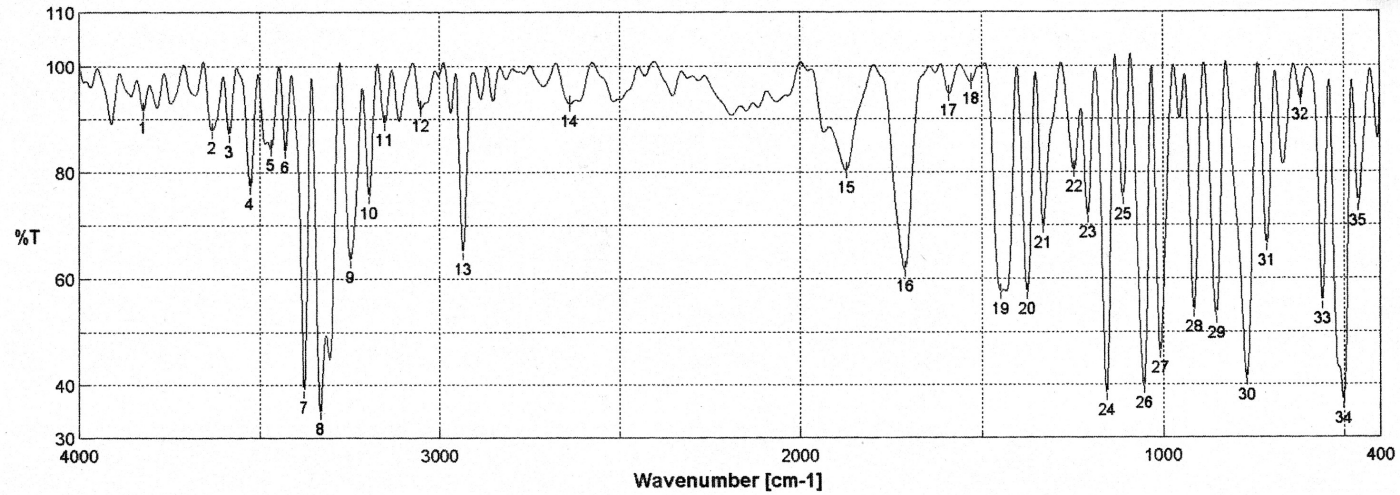
Sample: Crude sophorolipids

Method: KBr pellet. Interpretations of IR values were tabulated in table 27.

Table 27

IR values for sophorolipids obtained from *Candida bombicola* MTCC 1910

S.No	Peak No	Type of vibration	Wave (No cm^{-1})
1	8	O-H stretching	3330.46
2	13	C-H stretching	2934.16
3	16	C=O stretching	1714.41
4	17	C=C stretching	1590.99
5	24	C-O-C stretching	1157.08



Accumulation	16	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	16	Scanning Speed	2 mm/sec
Date/Time	11/21/2007 4:03PM	Update	1/30/2008 11:22AM
Operator	C.Geetha		
File Name	CB		
Sample Name	CB MTCC 1910		
Comment			

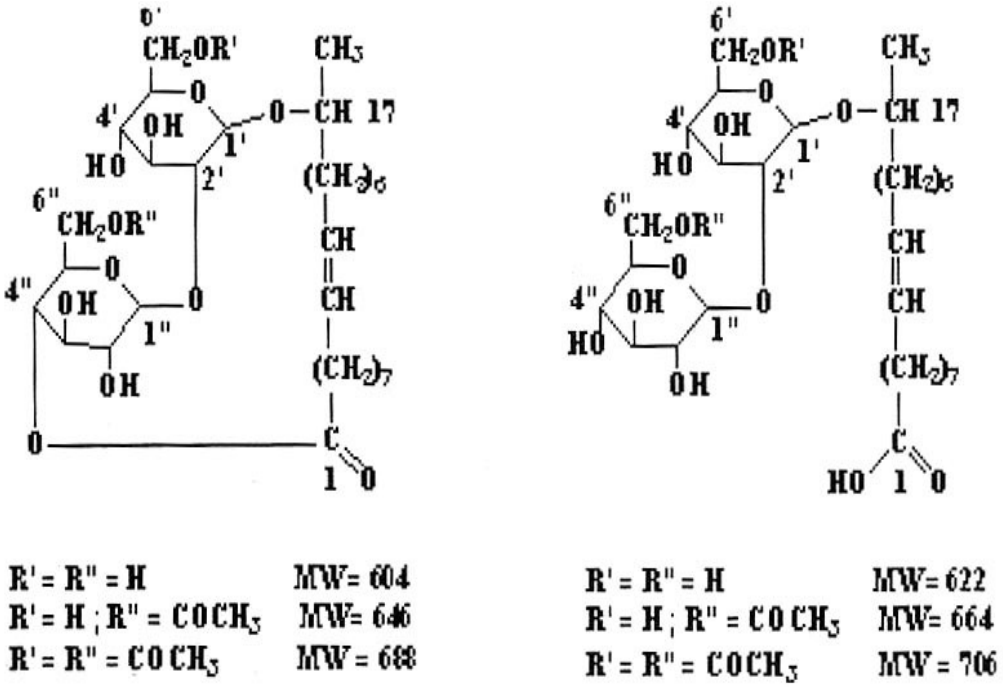
No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T
1	3823.19	91.7073	2	3629.37	87.863	3	3582.13	87.1247	4	3526.2	77.3147	5	3469.31	84.5343
6	3428.81	84.0645	7	3375.78	39.0007	8	3330.46	35.0233	9	3243.68	63.5393	10	3192.58	75.54
11	3150.15	89.3116	12	3052.76	91.9545	13	2934.16	64.9871	14	2639.11	92.8852	15	1873.51	80.3214
16	1714.41	61.7375	17	1590.99	94.6966	18	1530.24	96.9767	19	1447.31	57.4864	20	1375	57.457
21	1330.64	69.9145	22	1244.83	80.3832	23	1207.22	71.7797	24	1157.08	38.4026	25	1110.8	75.2612
26	1052.94	39.691	27	1007.62	46.3581	28	915.058	54.1062	29	854.311	52.9528	30	769.458	41.2687
31	713.533	66.5257	32	620.966	93.819	33	559.255	55.5325	34	501.401	36.8033	35	460.904	73.9011

Fig.26: IR spectrum of sophorolipids obtained from *Candida bombicola* MTCC 1910

5.12.2. LC-APCI-MS of sophorolipids obtained from *Candida bombicola* MTCC 1910[131]

Sophorolipid mixtures were separated by High Performance Liquid Chromatography (HPLC) with a waters 2690 separation molecule (waters co.miliford,MA,USA) using a 5cm x 2.1mm symmetry C18 3.5µm column coupled in series. The linear gradient elution was as follows: water: acetonitrile: (0.5% formic acid): acetonitrile (50:10:40) hold for 5min, to a final composition of water: acetonitrile (0.5% formic acid): acetonitrile :(40:10:50) over 25 min with a total running time of 50 min. The flow rate was 0.25ml/min$^{-1.}$

The effluent was connected to a micro mass ZMD mass spectrometer with an Atmospheric Pressure Chemical Ionisation (APCI) probe (waters co) set to the negative mode scan from m/z 900 at 2 seconds per scan. Corona pin voltage was tuned to 3.8kv, sample conc 20v and extraction conc 2v for detection of fragments and molecular ion.



Structures of lactonized and free acid forms of C18:1 sophorolipids.

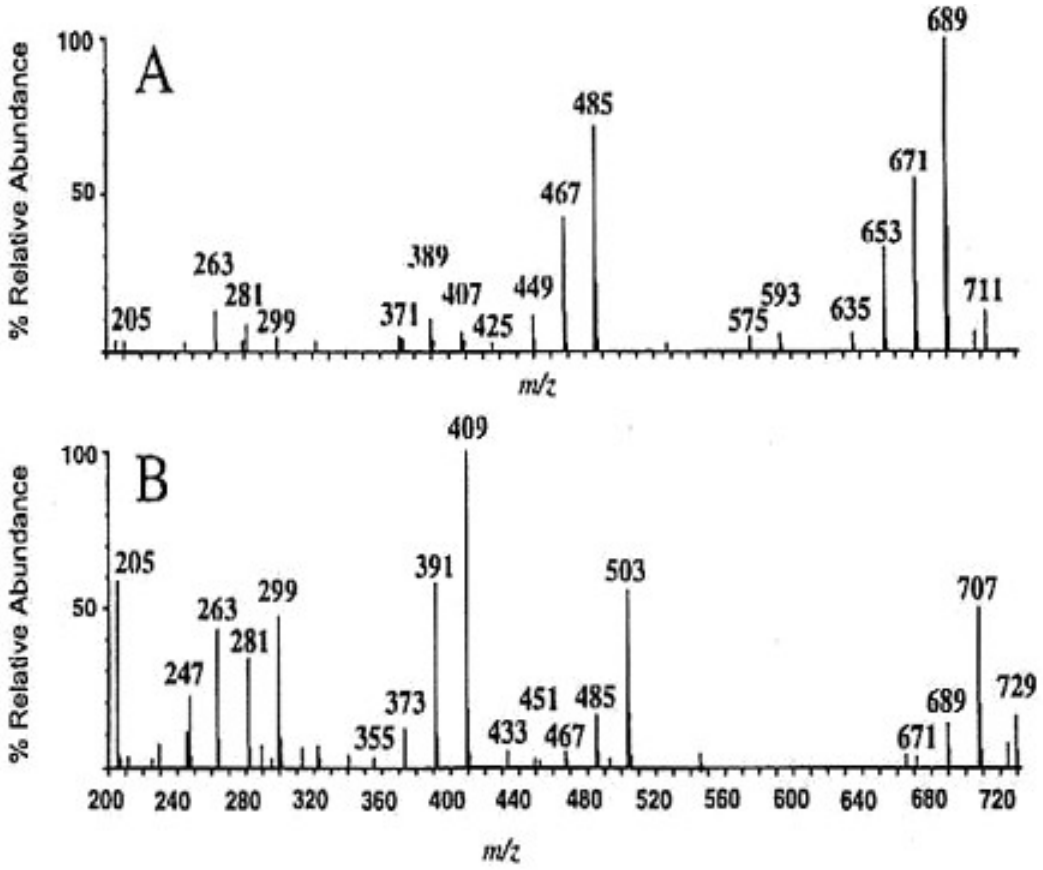


Fig. 27

Positive APCI – MS of C18:1 diacetylated sophorolipids collected from literarure. (A) Lactone form (B) Free acid

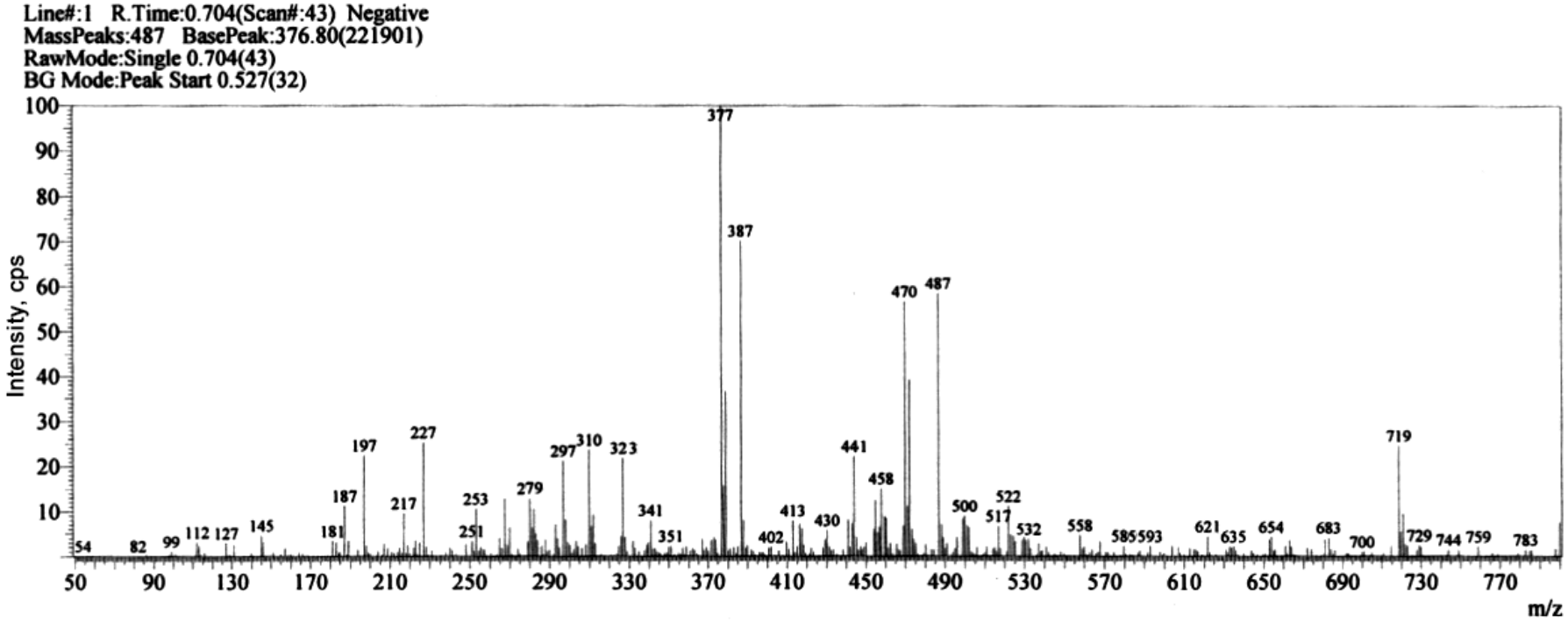


Fig.28

LC-APCI-MS (negative ion spectra) of sophorolipid obtained from *Candida bombicola* MTCC 1910

5.13. ANTIBACTERIAL AND ANTIFUNGAL SCREENING[145]

5.13.1. ANTIBACTERIAL SCREENING

Medium used in the study

Preparation of the Medium: Mueller-Hinton Agar

Mueller-Hinton broth gelled by the addition of 2% agar (bacteriological grade)

Ingredients of Mueller-Hinton broth

Casein enzymic hydrolysate	:	17.5 gL^{-1}
Beef infusion	:	300 gL^{-1}
Soluble starch	:	1.5 gL^{-1}
Final pH at 25°C	:	7.4±0.2

Preparation

The ingredients were dissolved in distilled water with the aid of heat; pH was adjusted to 7.2-7.6 using dilute acid or alkali.

Sterilization

15-20 ml of Mueller - Hinton agar was transferred to test tubes and sealed with non-absorbent cotton. It was then autoclaved at a pressure of 15 psi (120° C) for not less than 15 mins.

Organisms used

Escherichia coli NCIM 2911*, Bacillus subtilis* NCIM 2010 which were procured from National Chemical Laboratory, Pune and stored in Pharmaceutical Biotechnology Laboratory, College of Pharmacy, SRIPMS, Coimbatore-44. The strains were confirmed for their purity and identity by Gram's staining method and their characteristic biochemical reactions. The selected strains were preserved by subculturing them periodically on agar slants and storing them under frozen conditions. Fresh 24 hrs broth cultures were used for the study.

Working Conditions

The entire work was done using vertical laminar flow hood so as to provide aseptic conditions. Before commencement of the work, air sampling was carried out using a sterile nutrient agar plate and exposing it to the environment inside the hood. On incubation it was checked for the growth of microorganism and absence of growth confirmed aseptic working conditions.

Preparation of inoculum

The inoculum for the experiment was prepared fresh in Mueller-Hinton broth from preserved frozen slant culture. It was kept incubated at 37℃ for 24 hrs.

Drugs used	:	Sophorolipids (10mcg/disc)
Standard drug used	:	Ciprofloxacin 10 µg/disc
Vehicle used	:	Water

Screening procedure

Mueller - Hinton agar plates were prepared aseptically by pouring Mueller - Hinton agar prepared and sterilized previously to get a thickness of 5-6 mm, the plates were allowed to solidify and inverted to prevent the condensate falling on the agar surface. The plates were dried at 37℃ before inoculation. The organism were inoculated in the plates prepared earlier by dipping a sterile swab in the previously standardized inoculum, removing the excess of inoculum by pressing and rotating the swab firmly against the sides of the culture tube above the level of liquid and finally streaking the swab all over the surface of the medium three times, rotating the plate through an angle 60° after each application. Finally the swab was pressed round the edge of the agar surface. It was allowed to dry at room temperature, with the lid closed. The sterile disc containing test drugs, standard and blank were placed on the previously inoculated surface of the Mueller- Hinton agar plate aseptically. Petridishes were kept in the refrigerator for one hour to facilitate uniform diffusion and then plates were incubated for 18-24

hrs at 37℃. The zone of inhibition around the discs were observed and compared with that of the standard drug Ciprofloxacin. Results are shown in Table 28.

Table 28

Antimicrobial activity of sophorolipid from *Candida bombicola* MTCC 1910

Organism Used	Sample	Concentration Zone of Inhibition 10 mcg/disc
Escherichia coli NCIM 2911	Sophorolipid	-
	Ciprofloxacin (5mcg/disc)	29mm
Bacillus subtilis NCIM 2010	Sophorolipid	-
	Ciprofloxacin (5mcg/disc)	26mm

5.13.2. ANTIFUNGAL SCREENING

Medium : Sabouraud dextrose agar.

Ingredients

Mycological peptone : 10 gL^{-1}

Dextrose : 40 gL^{-1}

Agar : 15 gL^{-1}

pH at 25°C : 5.6 ± 0.2

Preparation

30 gms of Sabouraud dextrose agar was suspended in 1000ml of distilled water and boiled to dissolve the medium completely.

Sterilization

15-20 ml of medium was transferred to test tubes and sealed with non-absorbent cotton. It was then autoclaved at a pressure of 15 psi (120° C) for not less than 15 min.

Organism used

Candida albicans NCIM 3100 and *Aspergillus niger NCIM 545,* was procured from National Chemical Laboratory, Pune and stored in the Pharmaceutical Biotechnology Laboratory, SRIPMS, Coimbatore-44.

Drugs used : Sophorolipids (10mcg/disc)

Standard used : Fluconazole (10 µg/disc)

Vehicle used : Water

Screening procedure

Sabouraud dextrose agar plates were prepared aseptically by pouring Sabouraud dextrose agar prepared and sterilized previously to get a thickness of 5-6mm; the plates were allowed to solidify and inverted to prevent the condensate falling on the agar surface. The plates were dried at 25℃ just before inoculation.

The organism *Candida albicans* NCIM 3100*, Aspergillus niger* NCIM 554 *was* inoculated in the respective plates prepared earlier by dipping sterile swab in the inoculum, removing the excess of inoculum by pressing and rotating the swab firmly against the sides of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium three times, rotating the plates through the 60° after each application. Finally the swab was pressed around the edges of the agar surface. The inoculum was left to dry at room temperature with lid closed. Sterile discs containing the test and standard were placed in the petridish aseptically. The petridishes were incubated at 25℃ for 24-48 hrs. Observations were made for the zone of inhibition around the discs containing the drugs and compared with that of fluconazole. Results are shown in table 29.

Table 29

Antifungal activity of sophorolipid from *Candida bombicola* MTCC 1910

Organism used	Sample	Concentration Zone of Inhibition 10 mcg/disc
Candida albicans NCIM 3100	Sophorolipid CB	-
	Fluconazole (10mcg/disc)	21mm
Aspergillus niger NCIM 545	Sophorolipid CB	-
	Fluconazole (10mcg/disc)	20mm

5.14. ANTIOXIDANT STUDIES (FREE RADICAL SCAVENGING ACTIVITY BY DPPH ASSAY METHOD) [146]

Chemicals used

- DPPH (2,2-Diphenyl -1-Picrylhydrazyl) radical
- Methanol
- Ascorbic acid

Procedure

Stock solution of (1.0 mg/ml) test compounds prepared using 10% methanolic solution were diluted to get various concentrations of 0.5mM, 0.2mM, 0.1mM using methanol. After this 1.5ml of various concentration of test compound were added to 1.5ml of 0.2mm of DPPH radical in methanol (final concentration of DPPH was 0.1mm and final concentration of test compounds were 0.25mM, 0.1mM, 0.05Mm). The mixture was shaken vigorously and allowed to stand for 30min; absorbance at 517nm was determined and the percentage activity was calculated. Ascorbic acid was used as the reference standard.

Scavenging activity (AA%) = {[(Ab +As) - Am] / Ab} x 100%

Ab - absorbance of 0.1mM DPPH methanol solution at 517nm;

As - absorbance of various concentration solutions of test compounds at 517nm;

Am - absorbance of mixture methanol solution at 517nm.

Table 30

DPPH Free Radical Scavenging Activity of Test Compounds

Compound	Concentration mM	Absorbance			AA%= {[(Ab+As)-Am]/Ab}x100%
		As	Am	Ab	
Sophorolipid	0.25	0.0864	0.1750	0.2605DPPH (0.1 mM) =	65.98
	0.1	0.0347	0.1808		43.91
	0.05	-0.0143	0.2022		16.84
Standard (Ascorbic acid)	0.1	-0.0035	0.0101		94.77

6. RESULTS AND DISSCUSION

6.1. SCREENING FOR BIOSURFACTANT PRODUCING MICROORGANISMS

6.1.1. Hemolytic activity

Results

A total of 10 different species of *Candida* were examined for their respective response to an *in vitro* hemolytic test. Where the *Yeasts* species are incubated on sheep blood agar base for 48 h was used to evaluate the hemolytic activity.

Among the tested *Candida* species, *Candida tropicalis* NCIM 3120, *Candida tropicalis* NCIM 3122 demonstrated alpha (α) and Candida *bombicola* MTCC 1910 demonstrated beta (β) hemolysis at 48 h post inoculation, while all other species failed to demonstrate hemolytic activity after incubation for 48 h or longer.

Discussion

From observations made it is inferred that alpha and beta hemolytic activity may be a result of two or more different hemolytic factors sequentially produced by the *Yeast.* Thus it is tempting to hypothesize that the erythrocytes are destroyed by a two stage mechanism. First would be a partial destruction due to an alpha hemolytic factor(s) generated by the relatively young colonies of *Candida*. Thus, the metabolic end products of the first stage may serve as a catalyst to induce the secretion of a secondary hemolytic factor(s), beta hemolysin, leading to complete destruction of erythrocytes.

One more possible explanation for these observations may be the ability to produce a sophorolipid (biosurfactant) from *Candida* species which lead to hemolysis.

6.1.2. Determination of surface tension

Results

The important criteria for biosurfactant producing organism is the ability to reduce the surface tension of culture broth during fermentation by using medium containing 100 gL^{-1} glucose, 10 gL^{-1} yeast extract, 1 gL^{-1} urea and 40 mlL^{-1} oleic acid. Out of 10 different species of *Candida*, *Candida bombicola* MTCC 1910 was found to reduce the surface tension of culture broth below 40 dynes cm^{-1}.

Discussion

The reduction of surface tension of culture broth during fermentation of *Candida bombicola* MTCC 1910 is due to secretion of biosurfactant as extra cellular components

6.1.3. Sophorolipid biosynthesis

Results

In this study, five different screening media were used for identification of biosurfactant producing *Candida* species employing shake flask culture. A total of 10 different *Candida* species (obtained from NCIM and MTCC) were used in this study. Only *Candida bombicola* MTCC 1910 was found to produce sophorolipid (a glycolipid type) biosurfactant.

A maximum yield 56.3 gL^{-1} and 54.2 gL^{-1} of sophorolipid were obtained from *Candida bombicola* MTCC 1910 by using screening medium 1 and 2 respectively . Whereas by using screening medium 3,4 and 5 *Candida bombicola* MTCC 1910 produced a sophorolipid of yield 34.6 gL^{-1} , 41.3 gL^{-1} and 36.18 gL^{-1} respectively.

Discussion

From the study of sophorolipid biosynthesis *Candida bombicola* MTCC 1910 was found to produce sophorolipid in screening medium 1,2,3,4 and 5.

6.2. CULTURAL AND BIOCHEMICAL CHARACTERIZATION

Candida bombicola MTCC 1910 was selected as producer species and these species were subjected to cultural and biochemical characterization.

Results

6.2.1. Macroscopic morphology

On Sabouraud dextrose agar, *Candida bombicola* MTCC 1910 colonies are cream coloured with a slightly mycelial border. They are smooth, glabrous and yeast like in appearance.

It may produce a thin surface film and bubbles when grown in Sabouraud dextrose broth.

6.2.2. Microscopic morphology:

- **Stained with water - iodine solution**: Cells are oval shaped, colourless and reproduce by budding.
- **Stained with lactophenol cotton - blue solution**: Cells are oval shaped, blue colour and reproduce by budding. Revealed scars after budding.

6.2.3. Physiological test:

- Germ tube test is positive.
- Hydrolysis of urea is positive.

6.2.4. Fermentation test:

- Positive : D- Glucose, Dextrose, Sucrose, Lactose, Maltose.

6.2.5. Assimilation test:

- Positive : D- Glucose, Dextrose
- Negative : Sucrose, Lactose, Maltose

Discussion

The above methods provided correct identifications of *Candida bombicola* MTCC 1910.

6.3. OPTIMIZATION OF MEDIUM COMPOSITION

Results

Optimization of medium composition for sophorolipid production by *Candida bombicola* MTCC 1910 was carried out by shake flask culture method using 100 ml of basal medium in Erlenmeyer flask.

There was no sophorolipids produced from only carbon sources such as D-glucose, dextrose, sucrose, maltose and lactose (carbohydrates).Significant amount of sophorolipid was obtained in medium containing glucose (100 gL^{-1}), urea (10 gL^{-1}) and yeast extract (10 gL^{-1}) (nitrogen sources) from *Candida bombicola* MTCC 1910 by batch culture. But other nitrogen sources such as potassium nitrate, sodium nitrate, ammonium nitrate and peptone gave lesser yield of products. Among the lipid sources, oleic acid produced high yield of 63.4 gL^{-1} sophorolipid from *Candida bombicola* MTCC 1910. Medium containing soyabean oil produced lesser amounts of sophorolipids. The addition of 10% n-hexadecane and mineral salt to the medium of fermentation showed no significant improvement in yield. The optimized medium composition are given in Table

Discussion

When *Candida bombicola* MTCC 1910 species were grown on a medium containing only carbon sources, there was no yield from all *Candida* species. This showed that only carbon source is not enough to secrete sophorolipid (extracellular glycolipid biosurfactant). Significant yield of sophorolipid was obtained from *Candida* species in a medium containing glucose (carbon source), urea and yeast extract (nitrogen source). This yield could be improved significantly when oleic acid was added as a co-substrate to the medium. There was no improvement in yield when n-hexadecane and mineral salts were added. This is surprising because it is usually postulated that hydrocarbon (n-hexadecane) in a medium enhance the production of biosurfactant by bacteria and yeast.

6.4. OPTIMIZATION OF CULTURAL CONDITIONS

The cultural conditions such as pH, incubation period and speed of agitation were determined by shake flask culture method using 100 ml of basal medium in Erlenmeyer flask. The optimum pH for sophorolipid production was found to be at the pH 3.5. There was a drastic reduction in the yield when the pH is either lower or higher. From the study of incubation period, it is found that 7days incubation was an optimum incubation period for maximum yield of 86.5 g/L. All other incubation periods such as 96 h, 240 h and 312 h gave lesser yields. From the study of speed of agitation, higher yield at 200 rpm was obtained when compared with other speed of agitation.

Discussion

An optimum pH for sophorolipid production from *Candida bombicola* MTCC 1910 species was found to be pH 3.5. Any change to both lower or higher pH values caused an appreciable drop in sophorolipid yield. The 7 day incubation period was found to be optimum for maximum yield. A 200 rpm speed of agitation was found to be optimum for the producer species employed.

6.5. FERMENTATIVE PRODUCTION OF SOPHOROLIPID BY OPTIMISED MEDIUM AND OPTIMISED CULTURAL CONDITIONS

Results

The ideal condition for sophorolipids production in a shake flask culture methods with medium containing glucose-100 g/L, yeast extract-10g/L, urea-1g/L and oleic acid-40 ml/L has been studied. From this study Fed-batch-II fermentation method gave maximum of 98.50 g/L of sophorolipid from *Candida bombicola* MTCC 1910. Where as batch culture and Fed- batch-I fermentation method produced 86.49 g/L and 90.85 g/L of sophorolipids respectively.

Discussion

From the above study, *Candida bombicola* MTCC 1910 was found produce maximum yield in fed batch culture II (after 3 days, 40 g/L of glucose and 20 g/L of oleic acid was sequentially added for every 24 hrs till 144 h).In batch and fed culture II the yield was not significant. From this study, conclude that organisms required constant supply of carbon and lipid sources for maximum production.

6.6. CHARACTERIZATION OF SOPHOROLIPIDS

6.6.1. Determination of surface tension and CMC

Results

Characterization of sophorolipids was done by determining surface tension and CMC. The minimum surface tension of the crude sophorolipids from *Candida bombicola* MTCC 1910 was measured as 30.11dynes/cm and the CMC value of obtained sophorolipids was 30 mg/100.

Discussion

The sophorolipids obtained in the present study from the producer species are able to lower the surface tension of water below 30.11 dynes /cm and the CMC value of sophorolipid is found to be 30 mg/100ml. As per the literature reported a good surfactant can lower surface tension of water from 72 to 35 dynes/cm. The surface tension values of sophorolipids obtained in the present study was compared with commercially available chemical surfactants. From this it is confirmed that sophorolipids obtained in the present study acts as a good surfactant.

6.6.2. Scanning Electron Microscope (SEM)

Results

From the SEM analysis of sophorolipids (Fig.25a-25d) it was found that the resultant products (crude sophorolipids) are in flakes form. The surface and texture of sophorolipids are little rough and irregular in shape.

Discussion

Sophorolipids obtained in the present study from *Candida bombicola* MTCC 1910 was found to be in flakes form and amorphous in nature.

6.7. SPECTRAL ANALYSIS OF SOPHOROLIPIDS

6.7.1. FTIR

Results

An IR spectrum of crude sophorolipids was taken. From peak numbers 8, 13, 16, 17 and 24 the functional groups of sophorolipids O-H, C-H, C=O, C=C, C=C and C-O-C were confirmed respectively.

Discussion

The reference IR spectra of crude sophorolipids are not available in the articles. From the IR spectra, sophorolipids obtained from *Candida bombicola* MTCC 1910 was confirmed.

6.7.2 LC-APCI-MS of sophorolipids obtained from *Candida bombicola* MTCC 1910

Results

The negative APCI-mass spectra of the sophorolipid mixture obtained from *Candida bombicola* MTCC 1910 using oleic acid as a co-substrate are given in Fig. 28. Ions corresponding to protonated sophorolipid molecules (base peaks) was observed at m/z 621 which is due to acid type of sophorolipid. The base peak of the resultant product and the fragmentation ions are shown in table 31.

From acid type of sophorolipid m/z (622) the loss of 2 H_2O molecules ion gives peaks at m/z 585 and loss of $C_6H_{10}O_5$-H_2O produced another fragmentation peak at m/z 441. The loss of oxonium ion (B_2) produced another peak at m/z 327 and all other important fragmentation and their m/z values are shown in table 31.

The reference spectra (CID spectra) of sophorolipid obtained from *Candida bombicola* ATCC 22214 was shown in Fig.27. The base peaks and all other fragmentations obtained from *Candida bombicola* MTCC 1910 are compared with the reference spectra.

Table 31

LC-APCI-MS data of $(M-H)^+$ ions of sophorolipids obtained from *Candida bombicola* MTCC 1910

Molecular ions/ Fragment ions	m/z (relative intensity) Compounds	
	From reference[118]	From this study
M-H	621	621
M-H – $2H_2O$	585	585
M-H-$C_6H_{10}O_5$-H_2O	441	441
B_2	323	323
$C_{18}H_{35}O_3$ (=Y_0)	297	297
$C_{18}H_{33}O_2$	279	279
$C_{18}H_{31}O$	263	263

Discussion

The glycolipid surfactants studied most intensively are the sophorose lipids produced by strains from the genus *Candida* or *Torulopsis*. A scheme for the biosynthesis of *Candida bombicola* sophorolipid has been proposed by Asmer *et al*. (1988). Some works have been done on sophorolipid production from only *Candida bombicola* ATCC 22214. In the present study a new producer strain have been identified from *Candida* genus such as *Candida bombicola* MTCC 1910 which able to produce sophorolipids (glycolipid type) which are analyzed by Reverse Phase High Performance Liquid Chromatography method combined with Atmospheric Pressure Chemical Ionization Mass Spectral detection (LC-APCI-MS). The mass spectra of sophorolipid generated by APCI-MS gave ions similar

to those observed with CID-MS. From the LC-APCI-MS studies it was found that *Candida bombicola* MTCC 1910 produced 17-L-[(2'-O-β-glucopyranosyl- β-D-glucopyranosyl)-oxy]-9-octadecenoic acid (acid type) of sophorolipid from medium containing oleic acid in all different type fermentation process such as batch, Fed-batch-I and Fed-batch-II.

6.8. ANTIMICROBIAL AND ANTIFUNGAL STUDY

Results

The antimicrobial activity and antifungal activity of sophorolipids obtained from *Candida bombicola* MTCC 1910 species were evaluated by using disc diffusion method. No zone of inhibition was observed in antimicrobial studies on sophorolipids (10 mcg/disc), where as ciprofloxacin (10 µg/disc) produced 29mm zone of inhibition against *E.coli* and 26mm zone of inhibition against *Bacillus subtilis*. In antifungal study, sophorolipids (10 mcg/disc) did not produced zone of inhibition against *Candida albicans* and *Aspergillus niger*, whereas fluconazole (10 µg/disc) produced 21 mm and 20 mm zone of inhibition against *Candida albicans* and *Aspergillus niger*.

Discussion

From the results of antimicrobial and antifungal studies, it is concluded that sophorolipids isolated from *Candida bombicola* MTCC 1910 are do not possess any antimicrobial activity.

6.9. ANTIOXIDANT STUDIES

Results

From the results obtained by the screening of antioxidant activity, it was revealed that the reaction of sophorolipid with DPPH radical is in a concentration-dependent fashion i,e., when the concentration of the tested compounds were increased, the free radical scavenging activity also increased.

Discussion

Among the three concentrations studied the maximum free radical scavenging activity exhibited at concentrations of 0.25mM (66%).While rest of the concentrations showed free radical scavenging activity in the range of 17-44%.

Ascorbic acid which was used as the standard in this screening showed free radical scavenging activity of 94.77 % at 0.1mM concentration.

7. SUMMARY AND CONCLUSION

Candida bombicola MTCC 1910 was able to produce a maximum yield 98.5 gL^{-1} of sophorolipid only. The sophorolipids obtained were purified and characterized by evaluation of surface tension and CMC. The sophorolipid mixture obtained is identified by LC-APCI-MS. The identified sophorolipids is 17-L-[(2'-O-β-glucopyranosyl- β-D- glucopyranosyl)-oxy]-9-octadecenoic acid. This sophorolipid is used as a source for preparing a novel compounds of useful functionality such as glucoselipid-acid, 17-L-[(β-glucopyranosyl)-oxy-octadec-(9)-enoic acid)], [ω-1]-hydroxyfatty acid and (17-L-hydroxy-cis-9-octadecenoic acid), which are commercially not yet available and are difficult to prepare employing organic synthesis. This could be useful intermediate for polyester and macrocyclic lactone production. Besides their contribution as surface active agents, modification of sophorolipid opens a new field of special chemicals.

In recent years, microorganisms have found their application not only in the production of variety of metabolic products but also in the biotransformation of several chemicals. The genetically engineered microorganisms are also being used for the commercial production of some non-microbial products such as insulin, interferon, human growth hormone and immunological products like viral vaccines. Many complex bioconversions that are achieved by microorganisms, cannot be achieved by normal chemical means and this sophorolipid was obtained using *Candida bombicola* MTCC 1910. Chemically synthesized surface active compounds are widely used in the pharmaceutical, cosmetic, petroleum and food industries. However, with the advantages of biodegradability and production from renewable resource substrates, biosurfactants may eventually replace their chemically synthesized counter parts. So far, biosurfactants have only a few specialized applications because they have been economically uncompetitive. However, the use of cheaper substrates, optimal growth and production conditions coupled with novel and efficient multistep downstream processing methods and

the use of recombinant and mutant hyperproducing microbial strains can make sophorolipids production economically feasible. A judicial and effective combination of these strategies might, in the future, lead the way towards large-scale profitable production of sophorolipids and makes sophorolipids highly sought biomolecules for present and future applications as fine specialty chemicals, biological control agents, and new generation molecules for pharmaceutical, cosmetic and health care industries.

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